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STAPHYLOCOCCAL LEUCOCIDINS

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THE staphylococcus is known to produce at least two leucocidins: (*a*) The α-haemolysin, which attacks white as well as red cells. Its leucocidal action, however, is confined to cells of the rabbit. (*b*) The so-called Panton-Valentine (P-V) leucocidin, which attacks human as well as rabbit leucocytes, and is not haemolytic. This leucocidin is almost certainly the same as that originally described by van der Velde (1894; Denys and van der Velde, 1895). It has not always been clear that these two leucocidins are distinct (*e.g.*, Neisser and Wechsberg, 1901), but there is now little doubt that they are (*e.g.*, Panton and Valentine, 1932; Valentine, 1936; Wright, 1936; Proom, 1937; Jensen and Maaløe, 1950).

The present work forms part of an investigation into the nature of the P-V leucocidin and its relation to haemolysins and other extracellular substances produced by staphylococci. We have shown also that there is a third leucocidin.

METHODS

Strains of Staphylococci

A number of strains of staphylococci, both coagulase-positive and negative, were classified into 5 groups, as follows: (1) 60 coagulase-positive strains from human pyogenic infections, (2) 10 coagulase-positive strains from nasal carriers, (3) 5 coagulase-positive strains from cases of food poisoning, (4) 12 coagulase-positive strains from animal sources, and (5) 23 coagulase-negative saprophytic strains. Most of the work was done with strain V8 from group 1, received from Dr. F. C. O. Valentine of the London Hospital. It was isolated from a case of chronic furunculosis of 5 years' standing, was deficient in pigment, and produced high titres of P-V leucocidin.

Production of Leucocidin

For production of culture filtrate on the scale of litres the aerated column device of van Heyningen and Gladstone (1953*a*) was used. This supplied 4 l. of culture every 24 hr. For small-scale experiments (10 ml.) a modification of Monod's rocking T-culture tube was used (van Heyningen and Gladstone, 1953*b*).

The medium used was a modification of Gladstone and Fildes' (1940) CCY medium, as follows: Bacto Casamino acids, 160 g.; yeast diffusate, 1600 ml.; Na lactate 70 per cent

syrup, 113·6 ml.; Na glycerophosphate, 160 g.; $MgSO_4.7H_2O$, 0·16 g.; $MnSO_4.4H_2O$, 0·084 g.; 0·32 per cent (w/v) $FeSO_4.7H_2O$, 0·32 per cent (w/v) citric acid, 16 ml.; KH_2PO_4, 3·28 g.; $Na_2HPO_4.12H_2O$, 50·0 g.; distilled water to 8000 ml. The yeast diffusate was obtained by dialysing a suspension of 200 g. of Bacto yeast extract in 200 ml. distilled water against 1800 ml. distilled water in the cold overnight with constant agitation. For small-scale experiments the medium was distributed in 100 ml. quantities in flat 8-oz. (227-ml.) bottles and sterilized by autoclaving. For larger scale preparation the medium was sterilized in 8 l. volumes in 10-l. bottles by autoclaving at 15 lb. per sq. in. (1·1 kg. per sq. cm.) for 30 min.

The organisms for the inoculum were preserved in the dry state *in vacuo*. From this they were grown on tryptic meat agar slopes overnight at 37°. The slopes were then kept at room temperature and used for inoculation for a period of about 4 weeks, when they were discarded and fresh dry samples used. For the larger cultures the initial inoculum was 2 ml. of a visible suspension made from these slopes into 200 ml. medium in the column. This was grown overnight and the column then filled to the 2·2-l. mark. The first harvest (2 l.) was made after 10-hr. growth and the apparatus refilled with fresh medium. Thereafter 2 l. were removed every morning and evening, leaving 200 ml. to inoculate the fresh medium. Growth was measured in a photo-electric densitometer and expressed as mg. dry wt. per ml.

Cultures greater than 100 ml. were Seitz-filtered after centrifuging. Loss due to adsorption was negligible when 100 ml. or more of crude culture supernatant was passed through a 16-cm. pad. Smaller quantitites were tested without filtering. The leucocidin in the culture filtrate was concentrated by precipitation with ammonium sulphate at full saturation. Studies on the purifications of the leucocidin are in progress.

Assay of Leucocidin

We assayed leucocidin roughly by determining the minimal leucocidal dose and more accurately by titrating it against anti-leucocidin. In both methods we used a direct microscopical method of assessing death of the leucocyte. In addition it was also possible, though not convenient, to use a Ramon flocculation method of assay. Valentine's (1936) original anti-leucocidal K serum (B8760, Wellcome Research Laboratories) was no longer available, but we obtained from the Wellcome Research Laboratories 6 samples of antitoxin, 4 of which contained high titres of anti-leucocidin. One of these (KCP2029) was shown by Valentine (personal communication) to have a content of anti-leucocidin four times that of the K serum, and this was used in most of our assays. We gave this serum an arbitrary value of 100 anti-leucocidal units per ml. In addition it contained 340 units α-antitoxin and 12 units β-antitoxin per ml.

Microscopical "wet" method

Most of the work was carried out by observing the effect of leucocidin on living leucocytes in wet preparations. All glassware was scrupulously cleaned, since grease (probably the free fatty acid) is toxic to leucocytes. Slides, cover glasses and test tubes were immersed in concentrated sulphuric acid containing a few crystals of $NaNO_3$ at 60° and thoroughly rinsed. Slides and cover glasses were kept in alcohol. Tubes were dried at 37°, and not in the hot air oven, to avoid deposition of volatile grease. Pipettes were kept in sulphuric-bichromic acid mixture and rinsed with running tap water and dried with acetone as required.

Unless otherwise stated, assays were made with *human* leucocytes, generally from a single subject. Comparison between different subjects, however, showed no individual variation. When a drop of blood is allowed to clot on a grease-free cover glass, the polymorphonuclear leucocytes migrate from the clot and adhere to the glass. Harris (1953) showed that the clot and adherent red cells can be washed off without disturbing the leucocytes and that these remain viable for some hours. When the cover glass preparation is inverted over a drop of serum, or other suitable medium, on a slide and incubated, microscopical examination shows active amoeboid movements of the cells as they detach themselves from the glass surface.

A drop of blood was taken from a finger prick and allowed to clot on a cover glass and incubated at 37° for 30 min. in a moist chamber. Unless otherwise stated the clot and any adherent red cells were washed off with 0·5 per cent gelatine in normal saline (pH 7·6) and the preparation stored in this solution until required. Leucocytes remain viable and active for 2 hr. or more.

The same technique was used for *mouse* leucocytes, but is unsatisfactory with *guinea-pig* and *rabbit* leucocytes, since the clot fails to retract and migration of the leucocytes is scanty. Leucocytes were obtained from these animals after injecting saline intraperitoneally (about 200 ml. per kg.). After 18 hr. the animal was killed and the peritoneal cavity washed out with saline containing 0·3 per cent sodium citrate. The cells, of which about 90 per cent were polymorphs, were concentrated by light centrifugation and suspended in gelatine saline. A loopful of the suspension was placed on a cover glass at the time of assay.

For *sheep* leucocytes blood was obtained from a slaughter-house within 2 hr. of killing and lightly centrifuged. The buffy coat was removed and washed with 20 volumes of 0·5 per cent gelatine saline and finally suspended in an equal volume. One loopful of the suspension was placed on a cover glass at the time of assay. In addition to leucocytes it contained many red blood corpuscles.

Minimum leucocidal dose (*MLeD*).—A preliminary approximate estimation of the content of leucocidin in a culture filtrate was made by determining the minimum leucocidal dose. Serial 2-fold (or 1·41-fold) dilutions of the culture filtrate were made in gelatine saline. One drop of each dilution was placed on a slide (two dilutions on one slide) and the cover glass preparation of leucocytes inverted over it. The excess fluid was blotted off with filter paper and the preparation sealed with paraffin wax. Salts deposited on the surface of the cover glass were removed with damp filter paper. The slides were placed in an air incubator at 35° for 30 min. In order that they should reach temperature rapidly they were placed on a metal bar kept in the incubator. After incubation they were observed on a warm microscope stage with 1/12 in. (0·21 cm.) oil immersion lens under light phase contrast. The MLeD was the smallest amount of leucocidin to bring about the changes described under " Experimental ", and shown in Fig. 1–7, as evidence of death of the leucocyte. At this low level of leucocidin the end point was difficult to determine and the assay could only be approximate.

The test dose (*L*+).—The L+ dose is defined in the usual way as the smallest amount of leucocidin which when mixed with 1 unit of anti-leucocidin in a total volume of 1 ml. brings about the death of the leucocyte. Appropriate mixtures containing constant amounts of anti-leucocidin and logarithmically falling (20 per cent steps) amounts of leucocidin were made in tubes and 1 drop from each tube transferred to a slide, on which the coverslip with adhering leucocytes was inverted. The preparations were then sealed, incubated at 35° for 30 min. and examined as described above. A well-defined end point was obtained in the first mixture to contain an excess of leucocidin.

Normally the test was done at the 0·3 unit level, but with crude filtrates it was necessary to work at lower levels, *viz.*, 0·1 to 0·005 unit, since it was advisable to dilute these at least 1/5 in order to dilute out non-specific factors toxic to leucocytes. The L+ dose contains roughly 500 MLeD and consequently the end points of the titrations are still sharp at these low antitoxic levels.

P-V method

Valentine's (1936) method of assay was also used. Two ml. of saline containing 2 per cent sodium citrate were mixed with 2 ml. of fresh human blood and lightly spun for 10 min. The supernatant was removed and the red and white cells washed once with 10 ml. saline and re-suspended in 2 ml. saline. The usual leucocidin-antileucocidin mixtures were made and 0·1 ml. of the washed blood cells added to 1 ml. of each mixture and incubated at 37° for 1 hr. Films were made by streaking each mixture on a slide with a platinum wire, staining with Loeffler's methylene blue and examining with a 1/7 in. (0·36 cm.) oil immersion lens in a light microscope. According to Valentine, dead, but not live, leucocytes are mechanically destroyed in making the film, and the end-point is taken as that preparation showing no recognizable polymorphs.

Flocculation assay

The Lf dose was also determined by a modification of the semi-micro method of van Heyningen and Gladstone (1953c).

Assay of Haemolysins

α- *and* β-*Haemolysins.*—The haemolytic test doses (Lh) of these haemolysins were determined in the usual way, with 20 per cent differences in toxin in successive mixtures. For α-haemolysin, rabbit red blood cells were used and for β-haemolysin, sheep cells. Since the β-haemolysin

is a " hot-cold " haemolysin the toxin-antitoxin red cell mixtures were left at 4° overnight after incubation at 37°. When the haemolysin content was small the M.H.D. rather than the Lh was determined.

δ-Haemolysin.—Since this toxin is apparently not antigenic, only the M.H.D. could be determined. Human or horse red blood cells were used for these estimations.

Other Estimations

Hyaluronidase was estimated by the mucin clot prevention test of Tolksdorf, McCready, McCullagh and Schwenk (1949) on hyaluronic acid substrate (British Drug Houses), and the test controlled with testicular hyaluronidase (" Rondase ", Evans Medical Supplies, Ltd.).

Coagulase was estimated by the method of Gillespie, Devenish and Cowan (1939). Culture filtrates were mixed with an equal volume of human plasma diluted 1/10 and incubated at 37° in the water bath for 3 hr. and then for 18 hr. at room temperature.

Lipase was estimated by an antibody-combining test. Most commercial samples of α-antitoxic horse serum contain antilipase (Davies, 1954). To one of these, the anti-leucocidal standard serum KCP2029, an arbitrary unitage of 100 antilipase units was assigned. The lipase-antilipase mixtures were placed in capillary tubes, and on these were layered equal volumes of 10 per cent egg yolk extract in saline. Excess lipase was demonstrated by an opaque zone of highly refractile fatty acid particles at the junction of the fluids.

It was necessary to carry out the lipase antibody-combining assay at the 1-unit level in order to achieve a sufficiently high concentration of free enzyme at the end point.

For rapid surveys of lipase production by growing organisms, the diameter of the zone of clearing and blue coloration surrounding colonies plated on Nile blue fat plates was measured. These plates were similar to those used by Davies (1954) except that olive oil, emulsified in the agar by shaking on a Mickle homogenizer, was used in place of horse fat. Later, egg yolk plates (Gillespie and Alder, 1952) were used and the diameter of the zone of precipitated fatty acid surrounding the colonies measured.

Phosphatase production by growing organisms on phenolphthalein plates was detected by the method of Barber and Kuper (1951).

EXPERIMENTAL

Morphological Appearances of Human Leucocytes exposed to Leucocidin

Fig. 1–7 show the effect of adding a preparation of leucocidin (0·2 L+ per ml.) to human leucocytes. Changes are observed within minutes, the first being a loss of motility by the cell and a withdrawal of its pseudopodia. The granules lose

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EXPLANATION OF PLATES

Photographs taken under light phase contrast.

FIG. 1–7.—Changes in human polymorphonuclear leucocytes brought about by P–V leucocidin. Magnification × 600.

FIG. 1.—Normal leucocytes.

FIG. 2.—The same field as Fig. 1, 2 min. after the addition of 1 L+ P–V leucocidin per ml., showing withdrawal of pseudopodia.

FIG. 3.—The same field as Fig. 1, 4 min. after the addition of leucocidin, showing cytoplasmic protrusions.

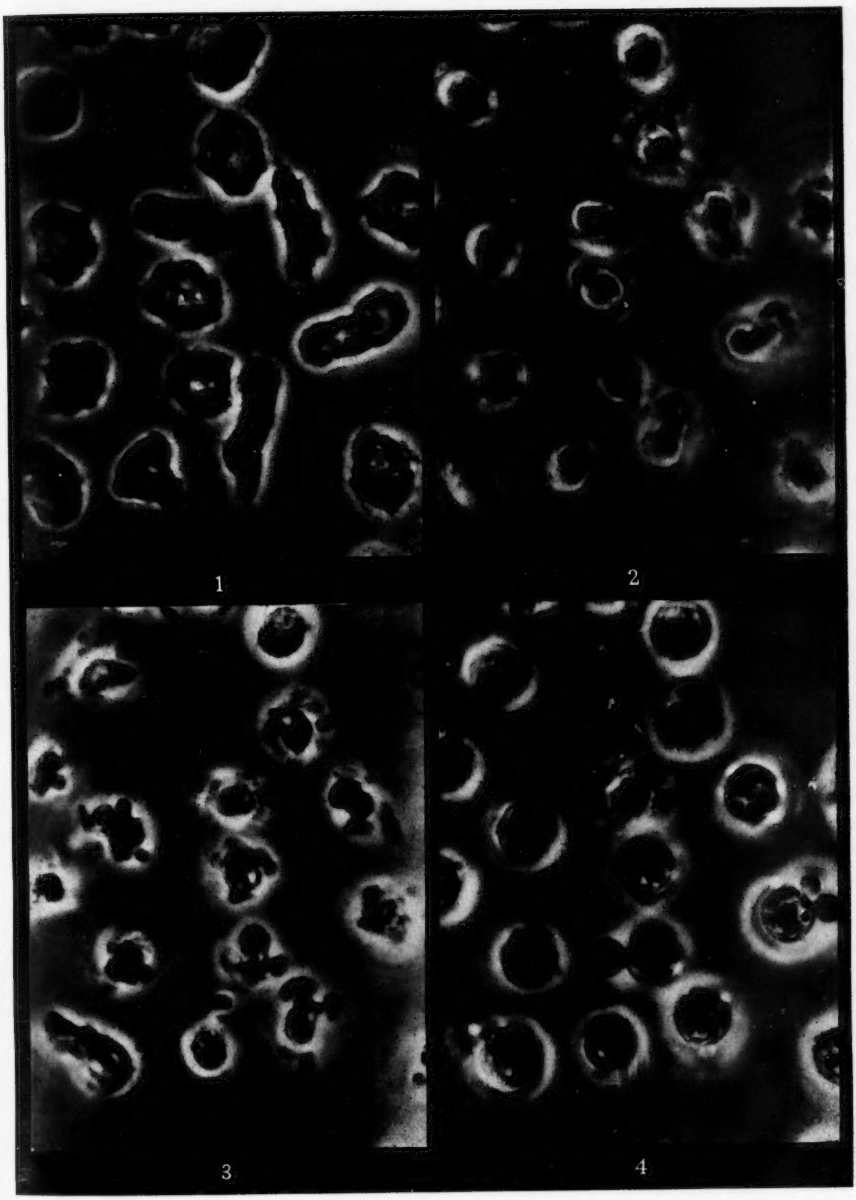
FIG.—4. The same field 6 min. after the addition of leucocidin. The nuclei are becoming prominent but still retain their polymorphic appearance. The cells are spherical and some cytoplasmic extrusions have been nipped off and show as spherical bodies.

FIG. 5.—The same field 8 min. after the addition of leucocidin. Many of the granules have disappeared. The nuclei are losing their polymorphic appearance and becoming spherical.

FIG. 6.—Fifteen min. after the addition of leucocidin. The granules have disappeared except for a few at the periphery of the cell. Nuclei are all spherical.

FIG. 7.—Eighteen hr. after the addition of leucocidin. The nuclei have disappeared and the cells are degenerate with foamy cytoplasm. No lysis has taken place.

FIG. 8.—The effect of δ-haemolysin on human polymorphs showing greatly swollen nucleus with the granules pushed to one side of the cell. " Signet ring " form. Magnification × 700.



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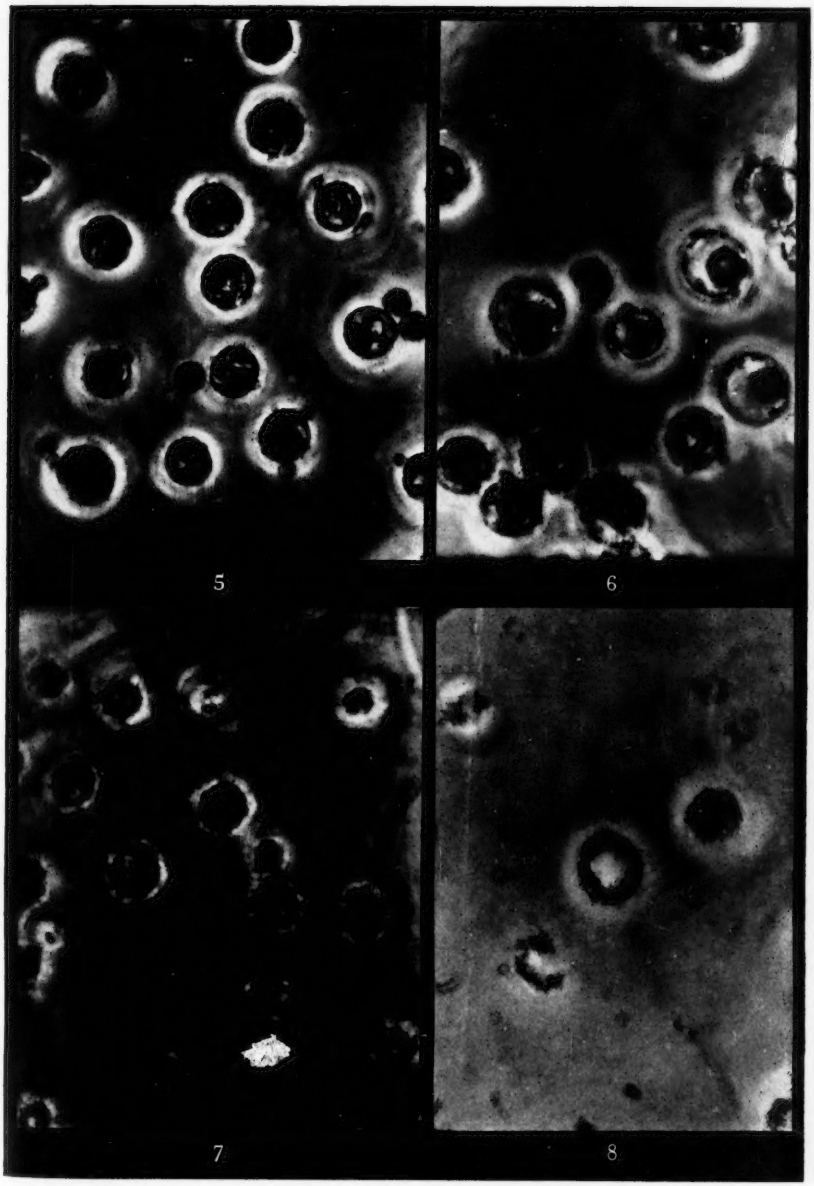

Gladstone and van Heyningen.

their orderly streaming and undergo Brownian movement. Rounded protoplasmic extrusions, quite unlike the " snail horn " pseudopodia, are violently put out and as violently withdrawn without any movement of translation of the cell as a whole (Fig. 3). Sometimes these protoplasmic extrusions are nipped off in the form of spheres which separate from the cell, taking granules with them (Fig. 4), but the cell as a whole does not disintegrate. After a minute or two these rounded extrusions cease to appear, the cell becomes spherical but not greatly swollen, and most of the granules disappear (Fig. 5). The remaining granules lose their Brownian movement and become closely applied to the wall of the cell. At this stage the nucleus becomes very prominent and heavily outlined. The next stage is the loss of the lobulated appearance of the nucleus which becomes spherical (Fig. 6). Cells at this stage stained by Sheehan and Storey's (1947) Sudan black method are entirely devoid of fat-staining granules. Eosinophiles show the same changes as polymorphs. The final stage is a gradual loss of the outline of the nucleus, the cell eventually becoming a practically empty vesicle containing a few granules (Fig. 7). Apart from the extrusion of parts of the cytoplasm as described above, no disruption of the cell ever occurs. We tested the lethal action of 0·1 N-HCl, 0·1 N-NaOH, 0·0033 M sodium oleate and distilled water and, although they are all lethal, in no case did they bring about these changes. With distilled water there was the same rounding of the cell, loss of streaming, and active Brownian movement of the granules, but there was swelling of the cell without disappearance of the granules and the nucleus became less obvious and showed no prominent outline.

Comparison between " Wet " and P-V Methods of Assay

A preparation of leucocidin concentrated by precipitation with $(NH_4)_2SO_4$ from a culture filtrate of strain V8 was assayed by both the " wet " and the P-V methods of estimation, the leucocidin being diluted in both ordinary and gelatine saline since Valentine (1936) used the former and we the latter. The usual clear end point was obtained with the " wet " method, distinguishing between 20 per cent steps in toxin concentration, irrespective of the dilution fluid used. With the P-V method the end point was much more difficult to determine ; in under-neutralized mixtures the polymorphs were still recognizable, although markedly degenerate. Moreover, mixtures giving a clear end point by the " wet " method contained many apparently normally staining cells in addition to those that were degenerate. The end point therefore appeared to be based on a subjective assessment as to what were normally staining and what were degenerate cells. Consequently we were unable to fix the end point within less than a 2-fold range and in subsequent work the " wet " method was used throughout.

Comparison between L+ and Lf Methods of Assay

Table I shows that when constant amounts of crude leucocidin were mixed with amounts of horse antiserum varying by 20 per cent steps over a 10-fold range, only a single zone of flocculation was obtained. The Table also shows that the first flocculating mixture contains about 40 per cent less toxin per unit of antitoxin than the first mixture to show excess free leucocidin, which suggests that the flocculation zone is due to the leucocidin, since the Lf dose is usually smaller than the L+ dose. This was confirmed by assays by both the Lf and the

L+ methods of 7 toxins against 4 antisera. The results of these are shown in Tables II and III.

TABLE I.—*Flocculation in Mixtures of Crude Leucocidin and Antiserum*

Each tube contained constant amounts of crude leucocidin solution, varying amounts of antiserum dilution and saline to constant volume. One drop of each mixture tested for leucocidal action by the "wet" method, the rest incubated at 37°.

Tube	Antitoxin units per ml.	Time of flocculation (min.)	Leucocidal action
1	72·5	78	0
2	58·0	61	0
3	46·4	35	0
4	37·1	24	0
5	29·7	14	0
6	24·9	11	0
7	20·3	9	0
8	16·2	11	0
9	13·0	22	+
10	10·2	28	+
11	8·7	>120	+
12	7·3	>120	+

TABLE II.—*L+ and Lf Values of Various Toxins against Standard Antitoxin KCP2029*

Toxin	L+ per ml.	Lf per ml.	Lf/L+
U_1	500	555	0·9
U_2	250	380	0·65
V_4C	312	641	0·48
	312	781	0·39
ZC	8	13	0·62
69B	40	62	0·64
A	20	25	0·8
IIA	111	178	0·62

TABLE III.—*Antileucocidin Contents of Antisera Estimated by L+ and Lf Methods*

	Units per ml.					
	Test toxin V8C			Test toxin ZC		
Antitoxin	L + method	Lf method	Difference	L + method	Lf method	Difference
CPP 68/36	144	128	+16	160	130	+30
CPP 80/63	128	100	+28	128	144	−16
CPP 81/B5	125	161	−36	160	156	+4
RK 5749/36	149	161	−12	160	130	+30

Mean difference between unitage by L + and unitage by Lf = 5·5.
Standard error mean difference = 232·12.
$t = 0·023$, 7 d.f.
Differences therefore are not significant.

The flocculation reaction requires a concentration of leucocidin of at least 5 Lf units per ml. and a corresponding amount of antiserum to give flocculation in a reasonable time, and since this was too wasteful of reagents the method was not routinely used.

The Production of Leucocidin

Little attempt has been made to determine the optimal conditions for leucocidin production since the medium did not appear to be critical provided it supported good growth of the organism. Except in the semi-solid agar medium used by Valentine (1936), relatively poor growth was obtained in stagnant cultures in tryptic heart broth or CCY. However, when these cultures were aerated by continuous shaking in air good yields of organisms and of leucocidin were obtained. Carbon dioxide was not only unnecessary, but in concentrations greater than 10 per cent was inhibitory to growth and to the production of leucocidin. Medium L described under " Methods " was found to be satisfactory and used throughout.

One litre of L medium inoculated with V8 strain was grown overnight in the large-scale aeration apparatus. Nine hundred ml. were then removed and the apparatus filled up to 1000-ml. mark with fresh medium. Samples were taken immediately and at hourly intervals, and the growth and leucocidin content estimated (Table IV). The concentration of leucocidin per unit weight of organism is at its maximum at the very beginning of growth and remains roughly constant for the next 18 hr. or more.

TABLE IV.—*Rate of Production of Leucocidin in Medium L* (10 *per cent Inoculum*)

Hours	Growth: mg. per ml.	L+ per ml. culture	L+ per mg. organism
0	1·4	0·16	—
1	1·4	0·16	—
2	1·75	0·32	0·18
3	2·6	0·35	0·13
4	3·2	0·64	0·2
5	4·4	0·8	0·18
6	4·8	0·8	0·17
7	5·1	1·0	0·2
9	5·2	1·0	0·19
10	5·3	1·0	0·19
18	7·5	1·0	0·16
42	8·8	1·2	0·14

Since the production of many bacterial toxins is improved when the iron content of the medium is reduced we thought it worth while to test whether this was the case with the leucocidin. The culture medium was de-ferrated by calcium phosphate precipitation in the usual way, but leucocidin production was unaffected.

The Effect of P-V Leucocidin on Rabbit, Mouse, Sheep and Guinea-pig Leucocytes

The work so far reported is concerned with human leucocytes. It was found that a leucocidin preparation had the same MLeD titre when tested against human and rabbit leucocytes, and that the cells underwent exactly the same changes. On the other hand, mouse, sheep and guinea-pig leucocytes appeared to be completely refractory to solutions containing 500 MLeD per ml. for human or rabbit leucocytes.

Relationship of P-V Leucocidin to Other Active Products

α-Haemolysin.—That the α-haemolysin is distinct from the P-V leucocidin is evident from the following :

(1) Strain V8 produced a poor zone of haemolysis on rabbit blood agar in 20 per cent CO_2 and no haemolysin was found in culture filtrates when grown under conditions giving 0·8 L+ leucocidin per ml. On the other hand, an old laboratory strain (N.C.T.C. 804) gave large zones of α-haemolysin on rabbit blood agar in 20 per cent CO_2 and culture filtrates contained 16 M.H.D. per ml., but less than 0·05 L+ leucocidin per ml. (2) A dried preparation of α-haemolysin (S883, Wellcome Research Laboratories) in a concentration of 2·3 Lh per ml. was found to have no action on human leucocytes. When tested on rabbit leucocytes, however, the cells were killed, but without the changes found with P-V leucocidin—beyond cessation of movement and withdrawal of pseudopodia they underwent little change. We have confirmed therefore the observation of Wright (1936) that α-haemolysin is lethal to rabbit leucocytes. A proviso must be made : since neither the production of γ-toxin by the strains mentioned above nor the content of γ-toxin in the α-toxin preparation was known, the conclusions relating to α-toxin may also apply to γ-toxin.

β-Haemolysin.—Culture supernatants of V8 in medium L containing 1 L+ leucocidin per ml. produced no hot-cold haemolysis of sheep red cells. A solution of β-haemolysin (S879, Wellcome Research Laboratories) containing 32 M.H.D. per ml. appeared to have no effect on human leucocytes. Thus the β-haemolysin and P-V leucocidin are separate entities. This observation is contrary to that of Flaum (1938), but confirms that of Wright (1936).

δ-Haemolysin.—The δ-haemolysin is the only staphylococcal toxin known to attack horse and human red blood cells (Marks and Vaughan, 1950). The V8 strain was tested for the production of this haemolysin by growing it on tryptic meat agar plates layered with cellophane in an atmosphere of 20 per cent CO_2 (Williams and Harper, 1947 ; Marks and Vaughan, 1950). The growth was washed off with 1·5 ml. saline per plate and the organism removed by centrifuging. The supernatant was tested on washed horse red cells, but no haemolysis was detected. It appeared, therefore, to contain no δ-haemolysin. When assayed for leucocidin it was found to contain 8 L+ per ml. ; conversely, V8 strain grown in medium L and producing 1 L+ P-V leucocidin per ml. failed to lyse horse red blood cells. Furthermore, δ-haemolysin and leucocidin have quite different properties. δ-Haemolysin is not specifically neutralized by staphylococcal antitoxic horse serum (Marks, 1951), whereas all of 6 samples of antitoxin obtained by us from the Wellcome Research Laboratories contained high titres of antileucocidin. δ-Haemolysin is stable to autoclaving at 115° for 20 min., whereas leucocidin is destroyed at 56° for 30 min.

Lipase.—Culture filtrates of V8 strain contain lipase, and all 6 samples of α-antitoxic horse serum tested contained antilipase. When lipase and leucocidin antibody-combining assays were carried out simultaneously on the same samples, no correlation was found between these two activities. For example, a crude culture filtrate containing 0·8 test dose of P-V leucocidin per ml. contained 0·3 test dose of lipase per ml., whereas an $(NH_4)_2\,SO_4$ concentrate containing 15 L+ per ml. contained no lipase. Moreover there appeared to be no correlation between lipase and P-V leucocidin production with other strains of staphylococci.

Colonies of some strains which produced no P-V leucocidin were surrounded by wide zones of opacity on egg yolk plates or clear blue areas on Nile blue-olive oil plates, whereas other strains producing no zones on these plates gave good yields of P-V leucocidin.

Phosphatase.—Another enzyme produced by coagulase-positive staphylococci is phosphatase (Barber and Kuper, 1951), which appears to be bound to the bacterial cell. It was found to have no relationship to the P-V leucocidin. Although all of 83 coagulase-positive strains produced phosphatase on phenolphthalein phosphate nutrient agar, no phosphatase could be demonstrated in liquid culture supernatants containing high titres of leucocidin. Moreover, some strains which failed to produce P-V leucocidin were phosphatase-positive on plates.

Protease.—Culture filtrates of strain V8 in medium L contain protease, active on gelatine and casein, and this is precipitated together with P-V leucocidin by $(NH_4)_2SO_4$. However, the protease and the leucocidin are evidently not identical since they can be separated by differential elution after adsorption on a column of calcium phosphate. This work is being continued.

Coagulase.—Although V8 strain is coagulase-positive it does not always produce coagulase, and failed to do so in cultures grown under optimal conditions for production of leucocidin; *e.g.*, 18 hr.-culture filtrates from medium L, containing 1 L+ leucocidin per ml. were devoid of coagulase. Conversely, strain "Newman" (N.C.T.C. 8178) produced high titres of coagulase in medium L, but no leucocidin. Although it is clear from these observations that P-V leucocidin is not coagulase, and that it is not produced by all coagulase-positive strains, none of 23 coagulase-negative strains produced any P-V leucocidin.

Hyaluronidase.—Hyaluronidase is produced by the staphylococcus, but it is clearly a separate entity from leucocidin since it may be absent from culture supernatants of V8 containing 1 L+ of leucocidin per ml.

Appearance of a New Leucocidin

So far all the work had been done with strain V8. Other strains of staphylococci from each of the 5 groups listed under "Methods" were now tested. The organisms were grown in T-tubes in medium L overnight and the supernatants of the cultures tested at a dilution of 1/5 on human leucocyte preparations. We were at once confronted with a new phenomenon. Whereas many of the coagulase-positive strains produced P-V leucocidin, certain of these, and a few of the coagulase-negative strains, seemed to produce an additional factor, for they not only destroyed the leucocyte but lysed it as well. The changes observed in the cell before lysis are quite different from those observed with P-V leucocidin. The effect seems to be mainly on the nucleus; it becomes swollen, loses its lobulated appearance and bulges the cell, pushing the granules to one side and giving the appearance of a signet ring (Fig. 8). The cell does not present the regular spherical appearance observed with P-V leucocidin, and the granules do not disappear. At this point the cell may or may not lyse. Indeed, a characteristic phenomenon is the lack of uniform reaction of individual cells. Some lyse rapidly, others remain like signet rings, and others show little change beyond withdrawal of pseudopodia and loss of movement. In this the reaction differs markedly from that obtained with P-V leucocidin, where every cell is uniformly affected. When lysis takes place Brownian movement of the granules

becomes more and more marked, the cell ruptures and the granules stream out. The enlarged nucleus is extruded either as a spherical or, more usually, as an egg-shaped body divided by a fine septum representing the demarcation of its original two lobes. The nucleus becomes less and less evident and finally disappears. A small mass of granular débris is all that remains of the cell.

This new lytic agent, or " leucolysin ", cannot usually be demonstrated in culture supernatants diluted more than 1/40. It does not diffuse through cellophane membranes ; is precipitated by saturated ammonium sulphate ; is stable to heating at 100° for 30 min. ; and is inhibited by relatively high concentrations of normal human and horse serum or by 1 per cent serum albumin. We have not found specific antibody to this lysin in any sample of staphylococcus antiserum, and are inclined to doubt whether it is antigenic.

It is clear that the " leucolysin " is distinct from the P-V leucocidin. At this point Dr. Elek and Dr. Lack directed our attention to a note by Jackson and Little (1956) to the effect that δ-haemolysin is lethal to human leucocytes. The properties of our " leucolysin " suggested that it might be δ-haemolysin : it is produced by most, if not all, coagulase-positive and by some coagulase-negative staphylococci ; it acts on red blood cells of all species of animals tested, including horse cells which are resistant to α- and β-haemolysins ; it is stable to autoclaving at 115° for 20 min. ; it is readily adsorbed on a Seitz filter ; it does not diffuse through dialysing membranes ; it is not specifically neutralized by antitoxin, but is inhibited by normal serum and serum albumin.

Culture supernatants rich in " leucolysin " were tested for δ-haemolysin. Serial 2-fold dilutions of the culture supernatants were made in gelatine saline and an excess of the standard antiserum added to neutralize any P-V leucocidin that might be present. Drops of each mixture were tested for their action on human leucocytes in the usual way, and then 0·5 ml. of a 3 per cent washed human or horse red blood cell suspension added to the remaining mixture and incubated at 37° for 1 hr. and then overnight at room temperature, and a rough visual estimation made of the degree of haemolysis.

Table V shows : (1) A correlation between " leucolytic " and δ-haemolytic activities ; (2) that the sensitivity of human leucocytes to " leucolysin " is of the same order as that of human or horse red blood cells to δ-haemolysin ; (3) that about 8 times as much δ-haemolysin was produced per unit volume of medium in medium L than on cellophane layered on tryptic meat agar, and that CO_2 was unnecessary ; (4) that " leucolysin " and δ-haemolysin are equally stable to heating to 100° for 30 min.

Further evidence that leucolysin is δ-haemolysin lay in the finding of complete correlation between the leucolytic action on human leucocytes and the haemolytic action on horse red cells in the supernatants of 45 strains. Since many of these strains also produced P-V leucocidin it was necessary to add excess anti-P-V leucocidin, or to heat the preparation at 56° for 30 min.

Leucolytic Synergism between β- and δ-Haemolysins

Marks and Vaughan (1950) noted that δ-haemolysin has a synergic effect in the haemolysis of sheep red cells by β-haemolysin. β-Haemolysin alone has no leucolytic action, but we found that it is capable of enhancing the leucolytic action of δ-haemolysin. A supernatant from a culture in medium L, containing 5 minimum leucolytic doses of δ-haemolysin per ml. was diluted to 1/20, well

TABLE V.—*Correlation between δ-Haemolysin and "Leucolysin" in Culture Supernatants of Various Strains of Staphylococci*

"Leucolysin" tested against human leucocytes; δ-haemolysin tested against 1 per cent suspension of human or horse red blood cells; 1 unit anti-P-V leucocidin added to each ml. dilution.

Organisms	Medium	Leucolytic and haemolytic activity* Dilution of culture supernatant 1/5	1/10	1/20	1/40	1/80
Group 1.—*Coagulase-positive pyogenic source*						
V8	Medium L	0, (0)	0, (0)	0, (0)	0, (0)	0, (0)
V8	Agar	0, (0)	0, (0)	0, (0)	0, (0)	0, (0)
F	,,	++, (++)	+, (+)	+, (+)	0, (±)	0, (0)
Foggie	Medium L	++, (++)	++, (++)	+, (++)	+, (±)	0, (0)
Newman	Agar	++, (++)	++, (++)	++, (+)	+, (±)	0, (0)
N1	Medium L	++, (++)	+, (+)	±, (±)	0, (0)	0, (0)
Group 2.—*Coagulase-positive, nasal carrier*						
56/3411	Medium L	++, (++)	++, (++)	++, (+)	+, (±)	0, (0)
Group 3.—*Coagulase-positive, food poisoning*						
1074	Medium L	++, (++)	+, (++)	±, (+)	0, (0)	0, (0)
Group 4.—*Coagulase-positive, animal sources*						
Z92	Agar	++, (++)	+, (+)	+, (+)	0, (±)	0, (0)
143/54	Medium L	++, (++)	+, (++)	+, (+)	+, (±)	0, (0)
42/54	,,	++, (++)	+, (+)	±, (±)	0, (0)	0, (0)
42/54	Medium L (heated 100°, 30′)	++, (+)	+, (±)	±, (0)	0, (0)	0, (0)
42/54	Agar	++, (++)	+, (+)	0, (0)	0, (0)	0, (0)
Group 5.—*Coagulase-negative*						
N.C.T.C. 6153	Agar	+, (+)	+, (+)	± (0)	0, (0)	0, (0)

All cultures in medium L (10 ml.) shaken in T-tubes in air for 18 hr.; agar cultures grown under conditions suitable for production of δ-haemolysin (Williams and Harper, 1947), *viz.*, 18-hr. growth on cellophane layered on tryptic meat agar in 20 per cent CO_2 and extracted with 1·5 ml. saline. (Supernatants from agar cultures were thus 8 times as concentrated as those from T-tubes.)

* In each column the signs on the left refer to leucolytic activity (++ = complete lysis, +, ± = changes in cell short of lysis), and those on the right, in brackets, to haemolytic activity (++ = complete haemolysis, + = 20–80 per cent haemolysis, ± = trace).

beyond its limit of leucolytic action. When β-haemolysin in a final concentration of 1·34 Lh per ml. was added to this dilution the preparation lysed human leucocytes strongly. The effect was not due to the enhancing action of proteolytic enzyme on δ-haemolysin (Marks, 1952), since our preparation of β-haemolysin contained no proteolytic enzyme.

Effect of δ-Haemolysin on Leucocytes of Other Species

Leucocytes from rabbits, guinea-pigs, mice and sheep were tested for their susceptibility to δ-haemolysin, and all except sheep cells appeared to be as susceptible as those of man. The experiments with sheep leucocytes were not

quite clear since it was impossible to rid the preparation of red cells, and these may have adsorbed the haemolysin. Marks and Vaughan (1950) have shown that the haemolytic action of δ-haemolysin depends on the concentration of red cells.

Estimation of P-V Leucocidin in the Presence of δ-Haemolysin

The leucolytic action of δ-haemolysin interferes with the assay of P-V leucocidin, unless the concentration of the P-V leucocidin is such that the δ-haemolysin can be diluted out, which is often the case. In other cases P-V leucocidin cannot be ruled out unless a test can be devised to detect it in the presence of δ-haemolysin.

We have confirmed Marks' (1951) observation that serum albumin inhibits the haemolytic action of δ-haemolysin, and have shown further that it inhibits its lytic action on human leucocytes. However, the concentration of human serum albumin used (1 per cent) also inhibited P-V leucocidin to some extent, and therefore we sought another inhibitor.

Cholesterol is known to inhibit certain haemolysins, *e.g.*, the oxygen-labile bacterial haemolysins and saponin (see Cohen, Halbert and Perkins, 1942). A suspension of 24 mg. cholesterol per ml. was made as follows: 25 ml. of a saturated solution of cholesterol in acetone at 37° was poured with continuous vigorous stirring into 500 ml. H_2O at pH 8. Coarse particles of cholesterol were strained off on cotton wool, the acetone was boiled off and the suspension reduced to about 1/5 of its original volume by boiling, to give the desired concentration of cholesterol, and finally re-filtered to remove coarse particles.

Various amounts of this suspension were added to culture filtrates containing δ-haemolysin and the mixtures tested for leucolytic and haemolytic action. Table VI shows that cholesterol, in a final concentration of 1·6–3·2 mg. per ml.,

TABLE VI.—*Effect of Cholesterol on the Leucolytic and Haemolytic Action of δ-Haemolysin*

(Excess anti-P-V leucocidin added in all cases.)

A. *Constant δ-Haemolysin (8 M.H.D., 8 MLeD per ml.), Varying Cholesterol.*

Cholesterol mg. per ml.	Leucolysis (human leucocytes)	Haemolysis (human red cells)
6·4	0	0
3·2	0	0
1·6	0	±
0·8	+	++
0·4	++	++
0	++	++

B. *Varying δ-Haemolysin, With and Without Cholesterol (Final Concentration 1·25 mg. per ml)*

Dilution of supernatant	No cholesterol		With cholesterol	
	Leucolysis	Haemolysis	Leucolysis	Haemolysis
1/5	++	++	+	++
1/10	++	++	0	+
1/20	+	++	0	0
1/40	+	+	0	0
1/80	0	+	0	0
1/160	0	0	0	0

The signs have the same significance as in Table V.

inhibits both the leucolytic and the haemolytic action of δ-haemolysin in a concentration of 8 M.H.D. or MLeD per ml. On the other hand, further tests showed that 6·4 mg. cholesterol per ml. had no effect on P-V leucocidin in a concentration of 1 L+ per ml. Cholesterol can therefore be used to inhibit δ-haemolysin in the presence of P-V leucocidin.

Survey of 110 *Strains of Staphylococci for Production of δ-Haemolysin and P-V Leucocidin*

Each of 110 strains from all 5 groups was grown in a T-tube in medium L and the 18-hr. culture supernatant tested in a dilution of 1/5; (*a*) without further addition, to detect any leucocidal or haemolytic activity; (*b*) *plus* 1 unit anti-P-V leucocidin per ml., to detect δ-haemolysin; (*c*) *plus* 2·4 mg. cholesterol per ml., to detect P-V leucocidin. One drop of each mixture was tested for its leucocidal action on human leucocytes, and to the remainder was added 0·5 ml. of 3 per cent washed horse red blood cells. The results of this survey are summarized in Table VII. This shows that P-V leucocidin and δ-haemolysin are randomly distributed amongst coagulase-positive staphylococci, irrespective of the source of the strains, except that only a few strains from animal sources produce P-V leucocidin. Although no P-V leucocidin is produced by coagulase-negative strains, many of these do produce δ-haemolysin.

TABLE VII.—*Distribution of P-V Leucocidin and δ-Haemolysin in* 110 *Strains of Staphylococci*

Group	Coagulase	Source	Number tested	Number producing P-V leucocidin*	Number producing δ-haemolysin*	Number producing both	Number producing neither
1	+	Pyogenic	60	31	39	21	11
2	+	Nasal carrier	10	2	9	2	1
3	+	Food poisoning	5	3	4	2	0
4	+	Animal sources	12	2	8	2	4
5	−	Saprophytic	23	0	7	0	16

* Supernatants from 18-hr. cultures in medium L tested at a dilution of 1/5. Hence numbers refer to strains producing more than 5 minimum leucocidal or haemolytic doses.

DISCUSSION

It is clear that there are at least three quite distinct staphylococcal leucocidins: (1) The α-haemolysin, sometimes known as the Neisser-Wechsberg leucocidin, active only on rabbit leucocytes without gross changes in their morphology; (2) the Panton-Valentine leucocidin, not related to any of the 4 haemolysins, or to any of the other known active products of the staphylococcus, and active on rabbit and human leucocytes with characteristic changes in morphology, but not active on guinea-pig, mouse or sheep leucocytes; (3) δ-haemolysin, active on the leucocytes of all species tested except those of sheep, and not only lethal to the leucocyte, but lytic as well. These leucocidins are randomly distributed amongst coagulase-positive staphylococci, irrespective of source, whether animal or human. δ-Haemolysin is also produced by some coagulase-negative saprophytic varieties.

From the differences between the changes in the leucocyte brought about by these leucocidins, it is evident that they act on the cell in different ways. The P-V leucocidin appears to act by altering the permeability of the membrane so that cytoplasm may be extruded through it without rupturing it. The rounded form assumed by the cell could be explained if the membrane (a non-rigid structure) were to become freely permeable to water, salts and protein. A spherical but greatly swollen form is also found when the cell is placed in distilled water, but this is the result of the increased osmotic pressure within the cell. P-V leucocidin does not cause a swelling of the cell, so that the spherical appearance cannot be due to increase in osmotic pressure. The disappearance of the granules due to P-V leucocidin can be demonstrated by their failure to stain for fat. We thought at one time that this might be due to the presence of lipase in the culture supernatants, but all attempts to equate lipase with P-V leucocidin have failed.

Lysis of the cell has never been observed when P-V leucocidin is free from δ-haemolysin. Where lysis has been observed (Proom, 1937) it is likely that δ-haemolysin was also present. Marks and Vaughan (1950) have called attention to the similarity between the lytic action of fatty acids on red blood cells and that of δ-haemolysin, and also to certain chemical properties common to these substances. They suggest that δ-haemolysin might be a lipoid-protein complex. Leucocytes are also lysed by sodium oleate, but only in the relatively high concentrations 0·01 M. At lower concentrations it is lethal to leucocytes, but there are no nuclear changes such as those found with δ-haemolysin. Recently, a lipid extracted from *Corynebacterium ovis* has been shown to be leucocidal (Carne, Wickham and Kater, 1956). It would be of interest to determine whether the changes observed in the leucocytes bore any resemblance to those observed with δ-haemolysin.

SUMMARY

Staphylococci produce three leucocidins: α-haemolysin active on rabbit polymorphs, the Panton-Valentine (P-V) leucocidin active on human and rabbit cells, and δ-haemolysin active on rabbit, guinea-pig, human and mouse cells. The P-V leucocidin was assayed by antitoxin-combining methods involving determinations of the L+ dose by microscopical examination using living human leucocytes, and the Lf dose by a Ramon flocculation method. A close correlation between L+ and Lf doses was obtained. The P-V leucocidin produces characteristic changes in the polymorph but never ruptures the cell. It cannot be equated with any of the four known haemolysins or with any other known active products of the staphylococcus. It is produced by 38 out of 87 coagulase-positive staphylococci tested, but not by any of 23 coagulase-negative strains. Staphylococci from human pyogenic infections and food poisoning more often produced it than strains from nasal carriers or from animal sources. δ-Haemolysin destroys polymorphs by an action on the nucleus, which becomes greatly swollen. In higher concentration the cell is lysed. Its leucolytic action on human leucocytes runs parallel with its haemolytic action on horse or human red cells. It is inhibited by cholesterol, so enabling P-V leucocidin to be assayed in its presence. It is formed by most coagulase-positive staphylococci irrespective of their source and by some coagulase-negative strains. The possible mode of action of these leucocidins is discussed.

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TRANSFORMATION OF NORMAL CELLS IN TISSUE CULTURE: ITS SIGNIFICANCE RELATIVE TO MALIGNANCY AND VIRUS VACCINE PRODUCTION

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THE pioneer work of Earle and his colleagues (Earle, Schilling, Bryant and Evans, 1954 ; Perry, Evans, Earle, Hyatt and Bedell, 1956) on the culture of tissue cells in suspension foreshadows an inevitable change from the present methods of virus vaccine production in primary culture, but this change will depend on the development of suitable cell lines capable of rapid multiplication and serial subcultivation *in vitro*.

The problem of serial cultivation of normal mammalian tissue cells has been studied by many workers (Parker, 1955 ; Gey, Bang and Gey, 1954 ; Swim and Parker, 1953 ; Zitcer, Cailleau and Kirk, 1954 ; Chang, 1955 ; Lahelle, 1955 ; Perry *et al.*, 1956 ; Berman and Stulberg, 1956). Many of these have used tissues of human origin and their success has been dogged by the spectre of malignant change in the cultivated cells. It is the fear of malignancy more than any other single factor which precludes the cell lines at present available from use in the production of virus vaccines, and this fear has been heavily underlined by the work of Moore, Southam and Sternberg (1956) on the tumour-producing capabilities of certain " normal " cell lines.

More recently workers at the Lilly Research Laboratories have reported work on the cultivation and virus spectra of cell lines derived from various animal species (Hull, Cherry and Johnson, 1956 ; Herberg and Hull, 1956) and on their cultivation in suspension (Cherry and Hull, 1956). The adaptation by Sheffield and Churcher (1957) of all three types of poliomyelitis virus to growth in rabbit cells of the embryo rabbit kidney line whose isolation is described below, opens up the possibility of vaccine production in cell lines of non-primate origin and demands a reassessment of the problem of malignant change and its attendant dangers.

During work in this laboratory on the serial cultivation of normal tissue cells from different animal species, certain of the cell lines cultivated have undergone dramatic changes in morphology and behaviour strongly suggestive of malignant transformation. These changes were comparable with, but not identical to, those reported by the earlier workers (Earle, 1943 ; Gey, Gey, Firor and Self, 1949 ; Goldblatt and Cameron, 1953 ; Parker, 1955) but resembled those recently observed by Berman and Stulberg (1956), Bell and Johnson (1956) and Jordan (1956). Moreover it is clear that some at least of the " normal " cell lines at present available were isolated by virtue of similar changes. In our experience the transformation has occurred with sufficient frequency to suggest that it may

be expected in a large proportion of cell lines derived from normal tissues and, in view of the growing importance of serial subcultivation, it is imperative that a fuller understanding of its true nature be obtained. It is our purpose to describe those lines in which transformation occurred, comparing them with the HeLa cell line of known malignant origin (Gey, Coffman and Kubicek, 1952), to discuss the possible significance of the transformation in relation to malignancy and to consider its implications as regards virus vaccine production.

MATERIALS AND METHODS

Culture method

Cells from normal tissues have been cultivated directly on glass surfaces using Dulbecco's technique of trypsin treatment (Dulbecco, 1952).

For preliminary digestion of tissues trypsin was used at 0·01 to 0·05 per cent concentration with or without the addition of 0·05 per cent versene; 0·1 per cent ficin or papain were also occasionally used.

Serial cultivation was carried out either in 8 cm. Carrel flasks or in large roller bottles of 100 ml. capacity.

Subcultivation was normally carried out when cultures became confluent, the cells being released from the glass by means of 0·01 per cent trypsin, 0·025 per cent versene or a combination of the two.

Medium

The standard medium used in most experiments was of the following composition:

Serum	10–15 per cent.
"TPY" supplement	5 „ „
Cysteine-HCl	0·02 „ „
Earle's saline to	100 „ „

The "TPY" supplement was made up as follows:

Tryptic meat broth	88 per cent.
Peptic digest of sheep's blood	2 „ „
Yeast extract	10 „ „

In some recent experiments lactalbumin hydrolysate 0·5 per cent (Melnick and Riordan, 1952) has been added to the standard medium.

Sera

All sera were Seitz-filtered and inactivated at 60° for 30 min. before storage at − 20°. Sera in current use were stored at 2°.

Antibiotics

Penicillin, 100 units/ml., was included in all media. Streptomycin, 100 γ/ml., and mycostatin, 20 units/ml., were also sometimes included.

Quantitative methods

The most satisfactory method for dispersing cells for counting was found to be by a combination of trypsin and versene, which yielded a suspension chiefly composed of single cells with only a few small clumps in which individual cells could easily be counted. This method was used in all the latter part of the work.

The cells of untransformed lines were found to be particularly adherent to each other and to the glass. It was frequently difficult to separate the cells from the glass and the tight clumps obtained could not be broken down by treatment with trypsin, however prolonged. As a consequence quantitative data are not available for most of the cell lines before transformation, since the versene method was not used in the earlier part of the work.

EXPERIMENTAL

Cell Lines Cultivated

The cell lines which underwent transformation did so after periods varying from 26 to 65 days from the time of primary culture. Of a total of 18 lines, obtained from adult and embryo rabbits, adult and embryo guinea-pigs, adult monkeys and a human embryo, and carried for over 30 days, 6 transformed. Of the remaining 12, 6 were cultivated for periods varying from 30 to 156 days, covering 6 to 20 passages, without change from the original cell type.

In Table I are listed the cell lines which will be considered in this paper. Of these the line MK1 alone represents the original untransformed cell type. Certain cultures of this line transformed at the 4th pass to give rise to the line MK2, but cells of the original type were carried on from unchanged subcultures. MK1 and MK2 therefore spring from the same stem.

TABLE I.—*Data on Cell Lines to be Considered*

		Tissue of origin	Cell type	No. of passages	Age of line (days)
Cell line	HLi1	Embryo human liver	T	56	330
	ERK1	Embryo rabbit kidney	T	80	420
	MK1	Adult monkey kidney	O	29	180
	MK2	" " "	T	20	180
	MK3	" " "	T	25	195
	MLu1	Adult monkey lung	T	9	180

T = Transformed.
O = Original untransformed.

*Human liver, HLi*1

Origin and isolation.—Liver from a 17-week human foetus was treated with trypsin and cultivated for 33 days in standard medium containing 10 per cent of human serum. At this time, after two passages, the cultures consisted of a mixed growth of strands of fibroblasts separating areas of epithelial cells. All but one of these cultures were lost at the 33rd day, through breakdown of the roller apparatus, having shown no signs of change in cell type.

The sole surviving culture was a primary liver culture in a Carrel flask from which a subculture had already been taken at the 16th day. A few cells had remained adherent to the glass and multiplied slowly over the next 10 days. On the 10th day, 26 days after primary culture, several small colonies of epithelial cells containing not more than 20 cells each, were noted in various parts of the culture. Mitoses were present in most colonies. By the next day the colonies had increased in size and number, and free-floating clumps of cells were present, in some of which mitoses could be distinguished.

Within 6 days the culture became semi-confluent, as much from the settling of free cells as from the spread of existing colonies. Subcultures, made originally from the free-floating cells and subsequently by trypsin treatment of the culture, were uneventful and the line is now in its 56th passage, having been cultivated for 330 days.

Morphology.—In appropriate media the cells closely resemble HeLa cells (Fig. 5 and 6), but morphology is influenced by the nature of the serum used.

In calf serum the cells grow as compact islands of flat polygonal epithelial cells firmly adherent to the glass and to each other. In human serum they are longer and narrower, forming spindles or elongated triangles with long cell processes ; unless overcrowded they are not closely adherent to each other but form an open reticulum rather than a sheet. Attachment to the glass is also loose so that many cells remain rounded and papillae are built up to break free into the medium. This behaviour also affects freshly inoculated suspensions, initial attachment often being poor in human serum and initiation of growth slow. Similar morphological changes in response to human and calf sera have been noted by Puck, Marcus and Cieciura (1956). Multipolar mitoses occur and may be of great complexity. Cell divisions yielding 3 or 4 daughter cells have been watched to completion.

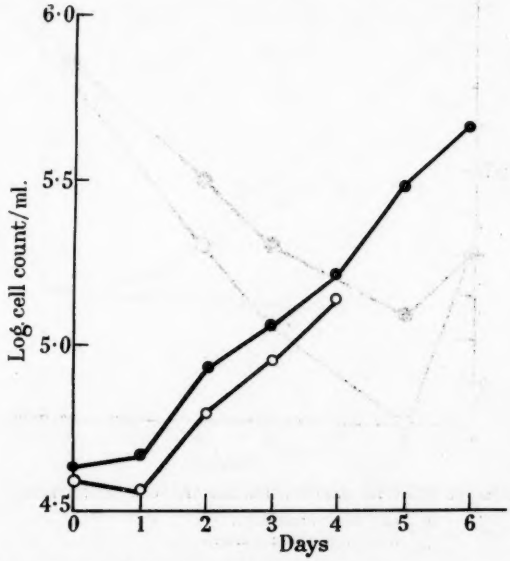

FIG. 1.—Multiplication of HLi1 in standard human serum medium. 3rd passage.
● counts using trypsin.
○ „ „ citric acid.

Multiplication rate.—In Fig. 1 are presented the results of an experiment at the 3rd passage in which 90 small roller bottles were inoculated with a calculated dose of 41,000 cells in 1 ml. of medium. Of these, 80 were placed in the roller drum after being left stationary overnight, while 10 were sampled immediately to determine the number introduced. Five were pooled and counted directly and 5 were treated by Earle's citric acid technique as modified by Parker, Healy and Fisher (1954). Thereafter 10 bottles were taken daily, 5 being treated with 0·01 per cent trypsin and five with citric acid. Haemocytometer counts were made on the pooled suspensions of each group. The citric acid method gave consistently lower counts than did trypsin but the slope in each case was the same and represented a mean doubling time of approximately 36 hr.

Effect of serum on multiplication.—The cell line was originally isolated in a medium containing human serum. In order to avoid difficulties attendant on the use of human serum attempts were made to adapt the line to growth in either horse or calf serum media. No success was obtained with the samples of horse serum tested but results with calf serum were encouraging. Morphology was altered as already described but growth occurred, with a doubling time slightly prolonged to 48 hr. In Fig. 2 are shown the results of an experiment comparing multiplication rates in media containing calf and human sera.

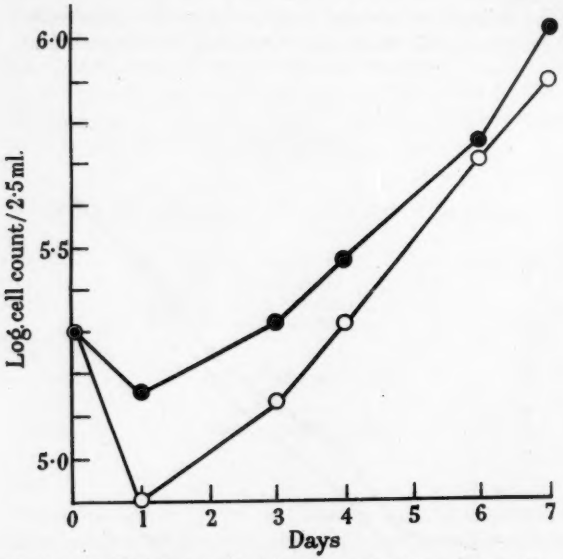

FIG. 2.—Multiplication of HLi1 in standard human and calf serum media. 34th passage.
● calf serum medium.
○ human serum medium.

The experiment was carried out in stationary cultures inoculated with 150,000 cells in 2·5 ml. of medium. Two cultures of each series were counted daily, the medium with unattached cells being discarded and the attached cells being brought into suspension by means of a mixture of trypsin and versene. The wide divergence in the 24-hr. counts illustrates the unavoidable loss of cells from the use of human serum, due to the failure of cells to attach to the glass, but those cells which gained attachment multiplied more rapidly in the human than in the calf serum medium, the doubling times being approximately 36 and 48 hr. respectively. Both the degree of attachment and multiplication rate were intermediate when a mixture of equal volumes of calf and human sera was used.

Despite satisfactory multiplication, it proved impossible to pass the cells in medium containing calf serum alone, but passage was possible in the calf-human serum mixture and the line was carried from the 4th to the 34th passage in medium containing this mixture. A further attempt to adapt the cells to calf

serum at the 35th pass was immediately successful, possibly due to the already long period of cultivation in the presence of this serum.

Effect of lactalbumin hydrolysate on the multiplication rate.—Fig. 3 shows the effect of the addition of 0·5 per cent of lactalbumin hydrolysate to the standard medium. The doubling time was reduced to 28 hr. from 42 hr. obtained in the standard medium. In the control cultures containing serum but no supplement the cells did not multiply and by the 3rd day were thin and spindly and obviously unhealthy.

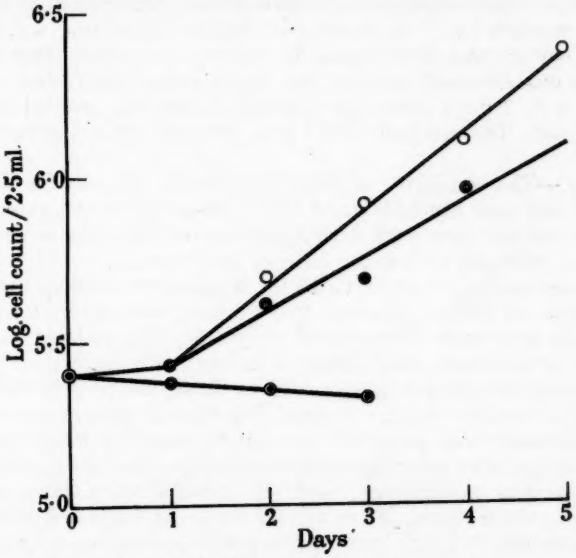

FIG. 3.—Multiplication of HLi1 in various media. 34th passage.

● standard calf serum medium.
○ " " " " + 0·5 per cent lactalbumin hydrolysate.
⊙ calf serum without supplement.

Lactalbumin hydrolysate is now included as a routine constituent of the medium for this cell line and has also been successfully used with other lines of cells.

Storage.—Cells of this line have been successfully stored for periods up to 30 days in a mixture of 20 per cent glycerine and 80 per cent calf serum at − 60°.

Stability.—No morphological or cultural change has occurred since the first isolation of the line apart from some diminution in the tendency to form free-floating clumps of cells. The multiplication curves shown in Fig. 1 and 3 were carried out on 3rd and 34th passage cells respectively.

*Embryo rabbit kidney, ERK*1

Origin and isolation.—One kidney from an embryo rabbit of 26 days' gestation was stripped of its capsule and the whole organ minced and treated with trypsin.

Primary cultures were set up in standard medium containing 10 per cent rabbit serum. The first cells to grow out were epithelial cells but these were soon followed by fibroblasts which predominated by the time the cultures became confluent on the 4th day. Subcultures were made with trypsin, some degree of dilution of the cells, representing a total of 1/10 over the series, being made at each passage up to the 7th, before which time the cultures had come to consist of fibroblasts only. In the 6th subculture, multiplication slowed markedly and it was feared that the line might be lost. At the 7th pass therefore the cells were concentrated from 4 to 2 large roller bottles, but, in spite of this, multiplication almost ceased. The cultures were left for 17 days without change of medium, and at the end of this time one culture was degenerate. In the other, a small plaque of about 30 epithelial cells was detected amongst the degenerating fibroblasts. The medium was renewed and, 2 days later, the enlarged colony was washed from the glass and subcultivated. The cell line ERK1 was built up without difficulty from this subculture.

Morphology.—The similarity of ERK1 to HeLa cells may be seen in Fig. 7. In rabbit and calf sera the cells grow firmly attached to the glass and to each other in sheets one cell deep with little tendency to heap up into papillae. Multinucleated giant cells and multipolar mitoses are frequent.

Effect of various sera.—The standard TPY supplement has been used throughout for the cultivation of this cell line but the effects of various sera have been tested. Horse and lamb sera were unsuccessful, very few cells sticking to the glass. In human serum attachment was better and moderate multiplication occurred, similar morphological changes being noted to those observed in the human liver cells. Growth in monkey serum was similar to that in human serum. In guinea-pig serum attachment was good but the cells developed a large amount of perinuclear granulation. Calf serum gave the best results, the cells growing as compact islands, of epithelial morphology, and the multiplication rate being at least equal to that in rabbit serum. From the 12th pass the line has been maintained in calf serum medium.

Multiplication rate.—The multiplication rate as determined in roller bottles is shown in Fig. 4. The doubling time was found to vary between 48 and 72 hr. in 15 per cent calf serum medium.

Storage.—ERK1 cells have been successfully stored for up to 180 days in a mixture of 20 per cent glycerine and 80 per cent calf serum at − 60°.

Stability.—No morphological or cultural change has occurred in this line since its isolation.

*Monkey kidney, MK*1

Origin and isolation.—Primary cultures were prepared in calf serum medium from the trypsinized kidney cortex of a rhesus monkey weighing 3·5 kg., and by the 15th day consisted of several layers of fibroblasts and epithelial cells. Subcultures were made by means of trypsin and by the 29th day the line was in its 4th pass and consisted predominantly of epithelial cells.

At this time 2 out of 4 cultures underwent transformation and gave rise to the line MK2, the remaining cultures being continued in direct series for 29 passages, during 180 days, as the line MK1. From about the 6th pass this line has consisted of epithelial cells only.

Morphology.—The cells are more delicate in structure than those of the transformed lines. They are larger and smoother in outline, with clearer cytoplasm ; the nuclei are larger and the nucleoli less dense with a delicate reticular structure (Fig. 10). The cells are so delicate in structure that the sheet of growth is almost invisible to the naked eye and can be distinguished only with difficulty by transmitted light. The boundaries between cells tend to be ill-defined and the cells are very strongly adherent to each other and to the glass. In growing cultures they tend to lie in whorls resembling those seen in primary culture (Fig. 9). When cells are isolated from their neighbours the cytoplasm spreads out over a large

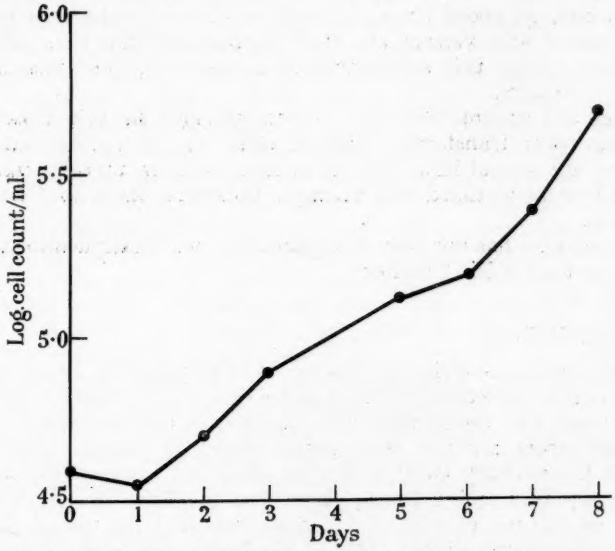

FIG. 4.—Multiplication of ERK1 in standard calf serum medium.

area of glass surface. Measurement of cell diameters in freshly-prepared suspensions indicates that the mean cell volume is significantly greater than that in the transformed lines, 4112 μ^3 as opposed to 3125 μ^3 (means of 100 cells. $P = 0{\cdot}03$) but comparison of the ratios so obtained with those of linear measurements in growing cultures shows also that the cells must be more thinly spread out on the glass. This fact probably contributes to the greater clarity of their cytoplasm.

Despite their resemblance to cells in primary culture, these cells are not normal. There is considerable irregularity in the size and shape of their nuclei and the numbers of nucleoli present ; multinucleated giant cells are common ; multipolar mitoses (Fig. 14 and 15) are very frequent and chromosome numbers high. The last two points will be considered in a later section.

Cultural behaviour.—One formal growth curve has indicated a doubling time of 60 hr. for this line, but counts on routine subcultures have shown that the doubling time is very variable and may be as little as 24 hr.

Storage.—No storage experiments have been tried.

Stability.—There has been a change, to be considered later, in chromosome numbers and in the frequency of multipolar mitoses from the 3rd to the 24th passage but the general morphology and growth characteristics of this line have not altered since early passage.

Monkey kidney, MK2

Origin and isolation.—The line MK2 arose by transformation in 2 of 4 cultures of MK1 at the 4th passage, 36 days after primary culture. Ten or more small areas of round cells and refractile epithelial cells arose and the cells rapidly heaped up into dense colonies about 1 mm. in diameter. Two colonies were washed from the glass, dispersed with versene and subcultivated, the line MK2 being built up without difficulty from this subculture. It is now in its 20th pass having been cultivated for 180 days.

Morphology and cultural behaviour.—In morphology the cells closely resemble HeLa and the other transformed lines of cells (Fig. 11). Their early cultural behaviour was like that of HLi1 but the tendency to heap up and break free from the glass has become modified with passage. Otherwise there has been no change in this cell line.

The doubling time has not been determined but the multiplication rate appears to be similar to that of MK3 (below).

Monkey kidney, MK3

Origin and isolation.—Primary cultures were prepared in 10 per cent monkey serum medium from the cortical kidney tissue of a 3·5 kg. rhesus monkey. Initial growth was poor, and the medium was charged to one containing 10 per cent of calf-human serum mixture, with improvement in the cultures. Of 7 lines derived from the primary cultures 6 were discarded between the 2nd and 5th subcultures, two undergoing foamy degeneration. The 7th line was used for experiments on the use of versene for subculture and the use of chick embryo extract as a medium supplement. One of 3 cultures of this line underwent transformation at the 4th pass 43 days after primary culture, the multiplication rate having slowed very markedly in this passage. The 2nd culture transformed 3 days later. In each case several colonies of transformed cells appeared simultaneously. The 3rd culture did not transform either in this or the next passage and was discarded.

Considerable difficulty was experienced in establishing the first subculture of the new cell type, but once this was achieved the line passaged easily and is now in its 25th pass after 195 days of cultivation.

Morphology and cultural behaviour.—The cells resemble those of the other lines (Fig. 13) except that they are sometimes more elongated with a tendency to show some resemblance to primary cultures of monkey kidney. This morphological appearance is sporadic in its occurrence. The cells tended in early culture to multiply in compact colonies similar to those of MK2 and showed the same tendency to heap up and break free, but as with MK2 this tendency has become modified with passage. Otherwise the line has remained stable.

In 15 per cent calf serum medium the mean doubling time is 36 hr. No storage experiments have yet been attempted.

Monkey lung, MLu1

Origin and isolation.—Small pieces of peripheral lung tissues, from the same monkey as provided the kidney tissue for the line MK1, were minced and treated with 0·1 per cent ficin. The primary culture, in 15 per cent calf serum medium, contained few viable cells but these multiplied steadily to give a confluent culture by the 36th day, at which time about 20 small areas of transformed cells were noted. The colonies were very similar to those appearing in the kidney cultures and were first seen on the same day. When they reached a diameter of about 1 mm. some were washed off the glass and subcultivated without difficulty.

Morphology and cultural behaviour.—The morphology was generally similar to that of HeLa cells (Fig. 8) but there was considerable pleomorphism in early passages and the cells had a more diffuse habit of growth than the other transformed lines. Mitoses were exceedingly numerous and many multipolar mitoses were seen, the cells multiplying very rapidly. At the 5th subculture, however, 4 cultures, which represented the entire stock of the cells, all started to degenerate simultaneously in a manner strongly suggestive of virus action. Two were subcultivated, and some of the cells attached and multiplied, but progressive degeneration started almost immediately. The medium did not appear to be at fault since it was similar to that used for other lines cultivated at the same time, and even in the degenerating cultures, healthy groups of cells showing mitoses could be distinguished. Eventually, however, nearly all the cells became affected and the line was feared lost. Samples of medium containing cell débris were transferred to cultures of HeLa, MK1 and MK2, but it proved impossible to transfer the degenerative process and its cause was never determined.

A few surviving cells from one culture were transferred to a slide-cell and very slow multiplication took place with concomitant diminution in the rate of degeneration. Despite the decrease in growth rate morphology was not altered and numerous mitoses were still present, but the mode of growth changed, the cells growing in tight knots where previously they had tended to be diffuse. This culture survived and the line has now been built up again, showing some tendency, in its 8th passage, to return to its original habit of growth but not to its original rate of multiplication. In its 10th, and most recent, passage the multiplication rate has greatly increased. No experimental work has been performed with this cell line.

Monkey testis

Testicular cultures were prepared from the same monkey as provided the tissues for MK1 and MLu1 and were similarly cultivated. Transformation occurred in the primary testis cultures at the same time as in the lung and kidney cultures, but transfer of the altered cells was not successful and no cell line was established.

Fig. 12 shows a photograph of one culture at the time of transformation and demonstrates the fact that the new cells arose among fibroblasts and seemed to be multicentric in origin.

Abnormal Mitoses and Chromosome Numbers

Multipolar mitoses and high chromosome numbers are characteristics commonly associated with malignancy in tissue cells. Multipolar mitoses occur in primary

culture of normal cells (ERK 2, Table II) but the impression was gained that in MK1 at least they had increased in frequency. Counts confirmed this impression, yielding the results shown in Table II.

TABLE II.—*Frequency of Multipolar Mitoses*

Cell line	Passage	Number of mitoses counted	Number of multipolar mitoses	Frequency per cent (to nearest 0·5)
HeLa	"A"*	500	102	20
	"B"	500	67	13·5
HLi1	49	637	43	7
ERK1	60	400	23	6
MK1	3	200	4	2
	24	500	115	23
	24	500	129	26
MK2	21	600	21	3·5
	22	500	17	3·5
MK3	14	100	7	7
	15	300	13	4
MLu1	10	500	22	4·5
ERK2	Primary	600	15	2·5

* Passage history of HeLa line is not known.

All mitoses were included in which it could be determined with certainty whether two or more poles were present in the spindle, and the counts cover all stages from early metaphase to early telophase. It will be seen that the highest frequencies of multipolar mitoses occurred in the HeLa and the untransformed MK1 lines, with little difference between the frequencies in the transformed lines. Further there had been a marked change in frequency in the MK1 line between the 3rd and 24th passages.

Chromosome counts on the current passages of these cell lines proved extremely difficult owing to the high chromosome numbers. Even using dilute medium for the spreading of chromosomes as suggested by Hughes (1952) and Hsu and Pomerat (1953*a*) only those cells with relatively few chromosomes could be counted with any pretensions to accuracy. The most that could be said with certainty is that all have mean chromosome numbers far in excess of 50. This contrasts strongly with the 3rd passage of MK1 in which the distribution of chromosome numbers is as shown in Table III. These counts cannot be related either to the haploid (24) or diploid (48) state of *R. macacus* as given by McClung (1939). The possible significance of these findings will be considered in the discussion.

Cause of Transformation

Although the habits of growth of the transformed cell lines are not identical, their morphological similarity and similar modes of origin justify the view that the separate episodes were examples of a single phenomenon. So far no experi-

TABLE III.—*Numbers of Chromosomes in 50 Unselected Mitoses of MK1 at 3rd Passage*

Number of chromosomes	Number of cells
10–14	1
15–19	6
20–24	9
25–29	10
30–34	11
35–39	3
40–44	2
45–49	3
Over 50	5
Total	50

mental work has been undertaken to determine its cause, but in Table IV are summarized the relevant data from which an indication might be obtained.

TABLE IV.—*Summary of Data on Cell Lines up to Time of Transformation*

	HLi1	ERK1	MK1	MK2	MK3	MLu1	MT
Treatment of tissue	T	T	T	T	T	Ficin	T
Treatment at subculture	T	T	T	T	T.V.	T	T
Medium	S	S	S	S	S,CEE	S	S
Serum	Hom.	Hom.	Het.	Het.	Het.	Het.	Het.
Day of transformation	26	65	—	36	43	36	36
Number of passage at transformation	0	7	—	4	4	0	0
Number of cultures transformed	1/1*	1/2	—	2/4	2/3	1/1	1/1
Number of original colonies	6±	1	—	10±	6±	20±	10±

T = Trypsin. V = Versene. S = Standard medium. CEE = Chick embryo extract. Hom. = Homologous serum. Het. = Heterologous serum.

* Numerator = Number of cultures transformed.
Denominator = Total number of cultures.

The following conclusions may be drawn :

1. Transformation was not induced by any specific factors in the preliminary treatment of the tissues or in the treatment of the cells at subculture, nor was it influenced by the use of heterologous as opposed to homologous serum in the medium.

2. Only some parallel cultures were affected.

3. It occurred in cultures of obviously mixed cell type or when fibroblasts only could be detected, but fibroblasts were always present when transformation occurred, and the possibility that they represent the parent type from which the transformed epithelial-type cells were derived is suggested by the ERK line and by the photograph (Fig. 12) of monkey testis. Jordan's observations (Jordan, 1956) also support this view.

4. The onset of transformation is not related to the number of transfers to which the cells have been subjected, nor is it obviously related to the total period of cultivation, but the coincidence of 26 to 65 days as the time range in our series and that of 21 to 65 days in Berman and Stulberg's (1956) series suggests that the total time in culture may in fact be of significance. We know of no instance in the literature in which a comparable change has occurred outside these limits.

5. There is a strong suggestion, particularly where transformations occurred in primary culture, that the origin of the new cells was multicentric. This suggestion is supported by observations by Pulvertaft on seminoma of the testis in plasma clot cultures in which the possibility of seeding by free cells could be discounted. Multiple areas of transformed cells appeared suddenly and simultaneously in several explants in the same culture tube (Pulvertaft, personal communication).

The summary in Table IV does not indicate any single factor common to all the transformed lines which did not equally affect MK1. One feature was, however, noted. At the time of transformation multiplication in each of the cell lines was slow or had ceased altogether. While this could not in itself be the cause of the change, it could assist in the emergence of a new rapidly multiplying cell type. Consequently it is possible that cells of the transformed type were present in the original suspensions, but took longer to adapt to conditions of growth *in vitro*, remaining unnoticed until slowing of the multiplication rate permitted their emergence. It seems unlikely, however, that islands of transformed cells, however small, could completely escape detection in cultures examined almost daily for up to 7 passages over 65 days ; nor is it easy to see why 17 days should have elapsed before the emergence of the ERK cells in a culture which had ceased to multiply and was starting to degenerate. Finally it would be odd if sudden adaptation to *in vitro* conditions should uniformly occur only after the majority of the cells had slowed in their multiplication rate. These events hardly fit the concept of an " adaptation ".

The change was in each case sudden and discontinuous, a new and stable cell type emerging in a single step from the old. The alternative possibility, therefore, that the change represents a true mutation seems more acceptable. Moreover if the origin of the new cells was in fact multicentric, this, and the fact that some only of the cultures were affected, suggests the influence of an external precipitating factor acting at near threshold level. It is possible that a changed physiological state, reflected by slowing of the multiplication rate, rendered the cells susceptible to such an influence.

Further speculation would be profitless at this stage, but one possibility must be considered. The foamy degeneration which terminated some of the monkey

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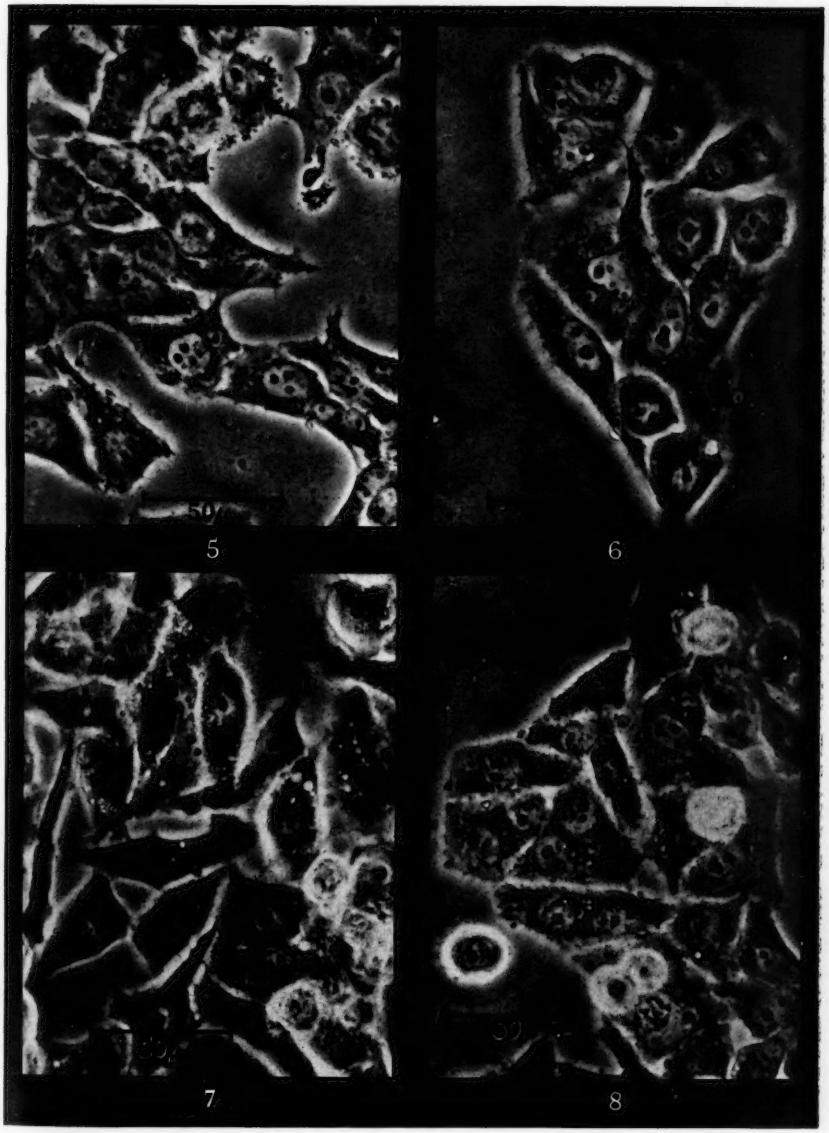
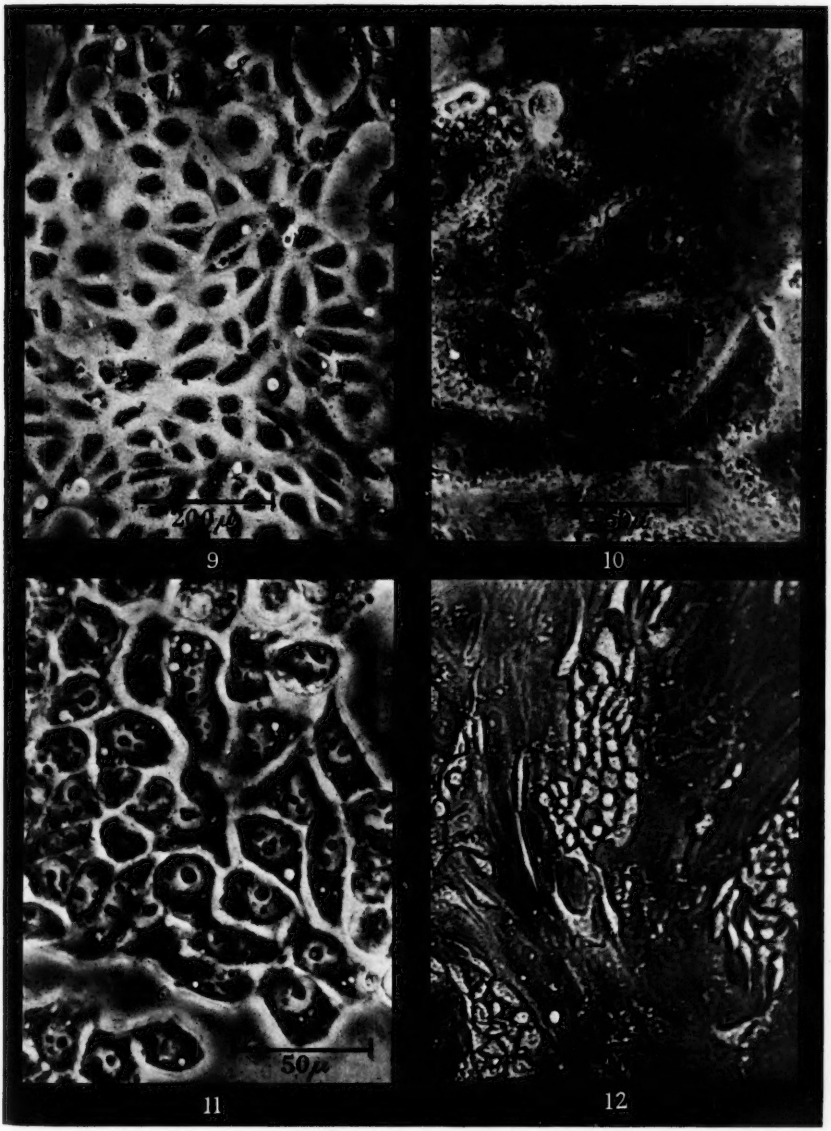
EXPLANATION OF PLATES

All photographs, except Fig. 12 and 15 were taken by phase contrast illumination.

FIG. 5.—HeLa cells.
FIG. 6.—Human liver, HLi1.
FIG. 7.—Embryo rabbit kidney, ERK1.
FIG. 8.—Monkey lung, MLu1.
FIG. 9.—Monkey kidney, MK1. Untransformed. Showing typical " whorled " appearance of growth.
FIG. 10.—Monkey kidney, MK1. Untransformed. Showing relatively clear cytoplasm, less dense nucleoli and large size of cells. The conditions of exposure and printing were identical with those of MK2 and MK3.
FIG. 11.—Monkey kidney, MK2.
FIG. 12.—Monkey testis. Culture in process of transforming, showing apparent origin of new cells among fibroblasts. The cells do not appear to be arising from a single centre. (Photographed by plain transmitted light.)
FIG. 13.—Monkey kidney, MK3.
FIG. 14.—Monkey kidney, MK1. Tripolar mitosis with early cleavage.
FIG. 15.—Monkey kidney, MK1. Multipolar mitosis. (Carnoy fixed and stained with thionin.)



Westwood, Macpherson and Titmuss.



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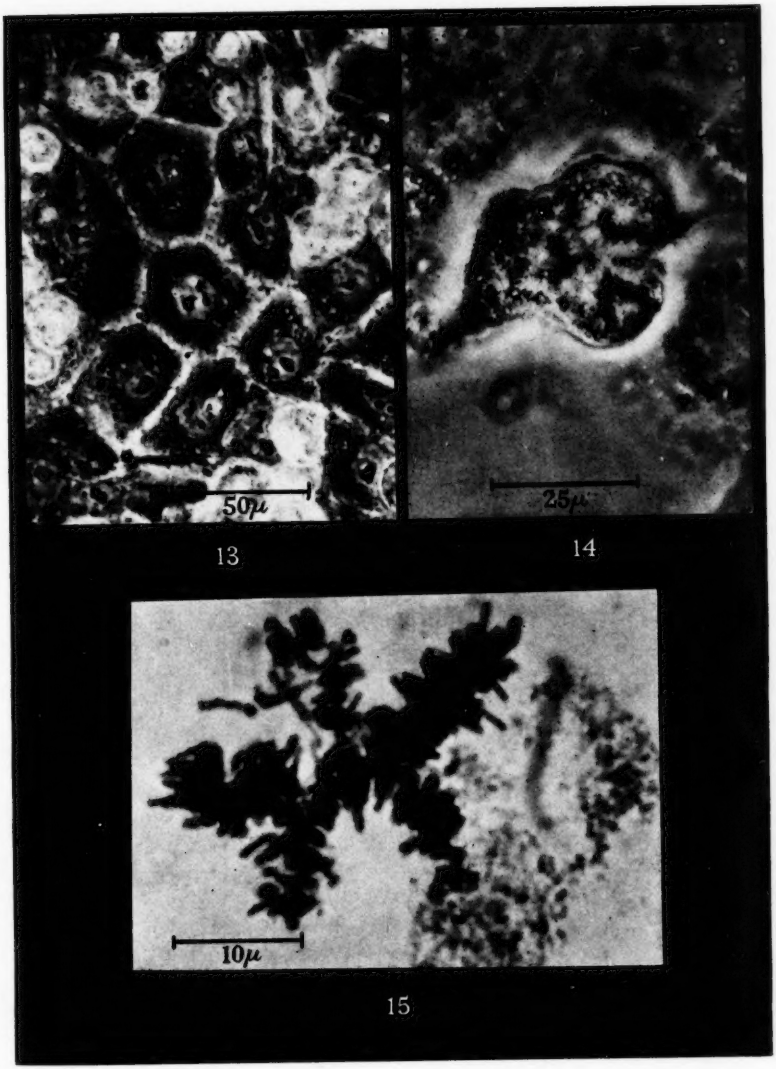

13 14

15

kidney cultures and the catastrophic degeneration of the monkey lung cultures suggest the presence of latent viruses in these lines. Whether latent viruses could precipitate transformation is problematical, but the possibility should not be overlooked.

Attempt to Induce Transformation

The two lines MK1 and MK2 appeared at first sight to be well suited to the induction of transformation by means of cell extracts, if such were possible.

Accordingly 40×10^6 cells of MK2 were concentrated into 5 ml. of calf serum medium, subjected to three cycles of freezing and thawing, and the cells and débris deposited by centrifugation at 2000 r.p.m. The supernatant was removed and added to an equal volume of calf serum medium to form Extract 1.

The deposit was re-suspended in 5 ml. of 0·01 per cent trypsin in Earle's saline and incubated for 15 min., by which time the cells were almost completely dissolved and the viscosity of the supernatant considerably increased. The solution was clarified by centrifugation at 2500 r.p.m. for 45 min., and approximately 100 γ of soybean trypsin inhibitor added in one drop of Earle's saline. An equal volume of double strength medium was then added to form Extract 2.

Both extracts represented fractions of the original 40×10^6 MK2 cells in 10 ml. of standard calf serum medium.

The following series of cultures were set up in slide-cells :

1. Control cultures containing no previous inoculum were inoculated with 2 ml. of each of the extracts in order to test for the presence in them of surviving viable cells of MK2.

2. The medium was removed from two 1/3 confluent cultures of MK1 cells in their 20th pass and replaced with Extract 1.

3. Two further cultures were similarly treated but the medium was replaced with Extract 2.

The cultures were followed for 6 days, the extracts being replaced by normal medium at the 4th day. No surviving MK2 cells were detected in the control cultures, while in the test cultures growth proceeded normally to give a thick sheet of normal MK1 cells at the 6th day. No signs of transformation were detected.

By itself this experiment may indicate no more than that the induction cannot be achieved in quite so naïve a manner, and is open to objection on the ground of choice of cell lines. It is possible that the cells of MK1, having once survived transformation in early culture, are refractory or, as indicated in the previous section, the presence of fibroblasts may be necessary. Consequently further consideration of the problem has been temporarily postponed.

Tumour Production in Animals

Preliminary experiments have been carried out, as part of an investigation to be reported elsewhere, on the ability of the transformed cell lines to produce tumours in animals. Rabbits, rats, mice and monkeys have been used, and results have shown the cells of all the lines to be capable of limited survival, and possibly some multiplication, in the subcutaneous tissues, but the tumours produced have been ascribable mainly to tissue reaction and have regressed after 8–10 days. So far no progressive changes have occurred in either homologous or heterologous hosts whether normal or cortisone-treated.

DISCUSSION

The possibility of using cancer cells for the production of virus vaccines for human use raises issues as much of a political as of a medical nature, and the political issues are less susceptible to the results of experimental investigation than are the medical. But the latter can be fairly accurately defined. In view of the filtration and inactivation procedures used in the preparation of a vaccine such as poliomyelitis vaccine, the danger of inoculating live cells can be discounted. The risk lies in the possibility of inducing malignant changes in the cells of the human subject by the introduction of an, as yet hypothetical, virus or non-living transforming principle analogous to that inducing change of type in the pneumococcus. Both these possibilities are susceptible of experimental investigation *in vivo* and *in vitro* as exemplified by our own attempt to induce transformation in the line MK1 by treatment with highly concentrated extracts of transformed cells from the same kidney tissue. Further, if virus vaccines can be produced in other than primate tissues, the question of specificity of virus or transforming principle must be considered.

In transformed cells derived from normal tissues the acquisition of malignancy is still not proven, although the work of Moore *et al.* (1956) is extremely suggestive as regards the Chang lines of human origin. The question of vaccine production, however, demands a closer definition of the problem of malignancy than is evident in most of the literature dealing with the production of malignancy *in vitro*. Malignancy as a property of cultivated cells can no more be defined in isolation than can virulence as a property of bacteria. In each case the property represents an interaction between a " parasite " and a specific host under defined conditions. The two cardinal criteria of malignancy are the invasion and destruction of normal host tissue and the production of metastases. But the ability of a cell line to fulfil these criteria, in an animal whose resistance has been destroyed by cortisone or irradiation, no more demonstrates malignancy than the ability of normally commensal organisms to multiply in the tissues of an irradiated host demonstrates virulence. Preliminary experiments with the cell lines described in this paper have shown their ability to survive and even multiply to a limited extent in the subcutaneous tissues of various hosts, but progressive changes have not so far occurred in either normal or cortisone-treated animals, even when the embryo rabbit kidney line was tested in rabbits.

Suspicion of malignancy in these lines arises from their morphology, which closely resembles that of the HeLa cell, from their ease of cultivation, their tendency to form multinucleated giant cells, their high chromosome numbers and the presence of multipolar mitoses. It is widely appreciated that the interpretation of such *in vitro* criteria of malignancy requires great caution (Moore *et al.*, 1956 ; Berman and Stulberg, 1956 ; Leighton, Kline, Belkin, Legallais and Orr, 1956) but the general feeling is that such cells must be regarded as malignant until proved otherwise. Yet ease of cultivation is largely a function of the medium employed, and multinucleated giant cells are frequently seen in primary cultures. The importance of multipolar mitoses is more difficult to assess. Since they occur in both normal and regenerating tissues their mere presence is not diagnostic of malignancy (Payling Wright, 1954) but their frequency may be of significance. In this connection they formed only 3·5–7 per cent of all mitoses in the transformed cells as against 13·5–20 per cent in HeLa and 23–26 per cent

in the untransformed MK1. Similarly polyploidy in normal tissues has been demonstrated by Hsu and Pomerat (1953*b*). Though the fate of polyploid cells in the tissues remains in doubt, in tissue culture they can undoubtedly divide to give fully viable daughter cells. When the possibility of endomitosis is also taken into account it is not surprising, therefore, that cells with high chromosome numbers should come to predominate in any cell line which is serially cultivated. This process is clearly shown by comparison of the counts obtained in the 3rd and 25th passages of MK1. On the other hand it is equally possible that the polyploidy and high incidence of multipolar mitoses in MK1 indicate malignancy despite the fact that the line has not transformed.

Earle (1943), Gey *et al.* (1949) and Goldblatt and Cameron (1953) have shown that malignant change may occur without mutation of the type here described, and the behaviour of Earle's control cultures indicated that malignancy may be acquired with minimum morphological change. By contrast, Parker (1955) has shown that both normal and malignant cells may undergo similar types of transformation in culture. Further, malignant cells, whether of *in vitro* or *in vivo* origin, do not conform in culture to a uniform cell type, whereas, in the present investigation alone, three different tissues from three different animal species gave rise to closely similar cell lines. If, however, the transformation represents, not malignant change, but mutation towards a less specialized cell type better suited to conditions of growth *in vitro*, this similarity would be less remarkable.

These considerations lead us to suggest that malignant change and transformation of the type here described are independent of one another, and that transformed cells are no more likely to be malignant than those of any other serially cultivable cell line. If the issue of malignancy must be decided on its own merits, then experimental justification for the use of such lines for the production of virus vaccines becomes possible.

SUMMARY

Six lines of cells, derived from the normal tissues of rabbits, monkeys and man, and serially cultivated for 180 days or more, are described.

One of the lines, derived from monkey kidney tissue, consists of epithelial cells which have retained their original morphology and cultural characteristics since first isolation, while the remaining five underwent transformation at periods ranging from 26 to 65 days after primary culture.

The transformed cells are epithelial in character and resemble each other closely but are clearly distinguishable from those of the untransformed line.

The origin and significance of transformations of this type are discussed with particular reference to implications of malignancy and the use of such cells for virus vaccine production.

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THE SERIAL PROPAGATION OF POLIOMYELITIS VIRUSES IN CELLS DERIVED FROM RABBIT EMBRYO KIDNEY

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THE introduction of human and monkey tissue cultures for the growth of poliomyelitis viruses (Enders, Weller and Robbins, 1949; Syverton, Scherer and Butorac, 1951) resulted in a rapid expansion of poliomyelitis research. Serious limitations, however, are still imposed by dependence on tissue explants and cell cultures of primate origin; regular supplies of monkeys are needed for tissue explants, while for the serial propagation of cell lines, such as strain HeLa, large amounts of human serum are usually required. Attempts to grow the viruses in tissue explants or in cell cultures derived from species other than primates have had little success hitherto. French workers have tested various tissues of many European and African mammals with uniformly negative results (Barski, Jezierski and Lepine,1954; Jezierski, 1955). Recently Kret (1955) has claimed that the three poliomyelitis virus serotypes can be grown in mouse carcinoma and sarcoma cells; this, if confirmed, represents a notable advance, but there are obvious objections to the use of malignant cells for many kinds of work. This paper reports the serial propagation of the three prototype poliomyelitis viruses in cultures of a new cell line derived from embryo rabbit kidney. The cell line, designated ERK, was isolated by Westwood, McPherson and Titmuss (1957) and has, in addition to its non-primate origin, the advantages of growing well in media devoid of human serum, being serially transmissible and yielding monolayer cultures if required.

MATERIALS AND METHODS.

Poliomyelitis virus strains

The three prototype poliomyelitis virus strains, Brunhilde, Lansing and Leon, were used in all the experiments. Seed pools of each strain were prepared from infected HeLa cell culture fluids and stored at −20°. The identity of the virus in each pool was confirmed by neutralization tests in HeLa cell cultures using standard type-specific monkey sera supplied by Dr. F. O. MacCallum of the Central Public Health Laboratory, Colindale.

HeLa cell cultures and infectivity titrations

Hela cell cultures were propagated and infectivity titrations performed by the methods previously described (Sheffield and Smith, 1956). To facilitate maintenance, the cell inocula in bottles used for titrations were reduced to 30,000.

ERK cell cultures and infectivity titrations

The cell strain ERK was obtained from Dr. Westwood. Details of its isolation, "transformation" and maintenance have been published (Westwood *et al.*, 1957). In this laboratory stock cultures were propagated in Pyrex babies' feeding bottles in a medium consisting of normal rabbit serum 10 per cent, tryptic digest broth 5 per cent, 5 per cent solution of lactal-

bumin hydrolysate in saline 5 per cent and Earle's solution 80 per cent with 100 units of penicillin and 100 μg. of streptomycin per ml. Stocks were subcultivated at approximately weekly intervals, the cells being dispersed by vigorous pipetting of the tissue culture fluid over the monolayer. The cell suspension was diluted to contain approximately 30,000 cells per ml. and dispensed in 10-ml. volumes in new bottles. The same suspensions were used to prepare cell cultures for virus infectivity titrations, but for this purpose 1-ml. volumes were dispensed into small bottles, 55 mm. × 20 mm., so that the titrations would be strictly comparable with those in HeLa cell cultures. After incubation at 36° for 48 hr. each bottle received an additional 1 ml. of fresh medium and an inoculum of the appropriate virus dilution contained in 0·1 ml. of 10 per cent fowl serum-saline. Five cultures were inoculated with each tenfold dilution of virus. Incubation at 36° was then continued, the cultures being examined daily for 7 days for cytopathogenic effect. The 50 per cent infectivity titres were calculated by the methods of Thomson (1947) and Lim (1954) and are expressed as the $\log_{10}$ of the reciprocal of the dilution of the inoculum.

Neutralization tests in embryo rabbit kidney cell cultures

Cultures similar to those used for infectivity titrations were employed in neutralization tests. Virus-antiserum mixtures were prepared to contain 100 TCD_{50} of virus per inoculum and were allowed to stand at room temperature for 1 hr. before being used to inoculate cultures. Standard type-specific monkey sera suitably diluted were used throughout.

EXPERIMENTAL

The Cytopathogenic Effect of Prototype Poliomyelitis Viruses in ERK Cell Cultures

Titrations of the three prototype poliomyelitis virus seed pools were performed in parallel in ERK and HeLa cell cultures. For these and all subsequent titrations one dilution series of each virus strain provided inocula for both types of cell culture. All three virus strains were found to have a cytopathogenic effect in ERK cell cultures closely resembling that seen in HeLa cells. Cultures infected with large virus inocula develop areas of dense rounded cells within 18 hr.; cultures with small inocula may require up to 6 days' incubation before degeneration appears. Once evident, this cytopathogenic effect spreads in 24 hr. to involve all the cells in a culture ; a further 24 hr. suffices for the cells to become detached from the glass. As shown in Table I there is no significant difference in the sensitivity of the HeLa and ERK cell lines. The ERK line, therefore, is quite as suitable as HeLa cells for the detection and titration of poliomyelitis viruses or for the assay of neutralizing antibody.

TABLE I.—*Log Titres of Poliomyelitis Virus Seed Pools in HeLa and ERK Cell Cultures*

	Brunhilde		Lansing		Leon	
	HeLa	ERK	HeLa	ERK	HeLa	ERK
Before serial passage expt.	6·1	6·1	3·8	3·7	4·5	4·7
After serial passage expt.	5·9	6·1	2·5	3·5	4·5	5·1

The Serial Propagation of Poliomyelitis Viruses in ERK Cell Cultures

Before a new host system can be accepted for regular use in poliomyelitis work it must be proved capable of supporting the serial propagation of virus strains of each of the three serotypes. Also the virus titres attained by cultivation in the new cells should be such as to allow the production of potent virus vaccines and concentrates. The behaviour in ERK cells in these respects was investigated.

The virus seed pools were re-titrated in HeLa and ERK cell cultures. The results were similar to those of the previous titration. On this occasion all ERK cell cultures showing cytopathogenic effect were transferred to a refrigerator as soon as cell detachment was complete. At the end of the 7-day titration period one of the ERK cell cultures of each virus type infected with a minimal infective dose was used for passage. The tissue culture fluids were centrifuged free from cells and titrated for virus content in HeLa and ERK cell cultures. This was repeated through four successive passages. The fluids from cultures of the fourth passage infected with minimal infective doses were used to infect ERK cell cultures in babies' feeding bottles. An inoculum of 0·1 ml. of undiluted cell-free fluid was used for each bottle. Complete detachment of the cells occurred within 72 hr. The fluids were harvested, centrifuged and ampouled in 1·0 ml. volumes for storage at −20°. These three pools of poliomyelitis virus grown in ERK cells were then titrated in HeLa and ERK cell cultures.

Table II shows the results of infectivity titrations obtained in these experiments. The infectivity of each Brunhilde fluid used for passage was almost identical for both types of cell culture. The effect of propagation in ERK cells on the Lansing and Leon viruses, however, was to make them relatively less virulent for HeLa cells than for ERK cells. This was more marked with the Lansing virus fluids which were, on the average, two hundred times less infective for HeLa cell than for ERK cell cultures. An average difference of 25-fold was observed for the Leon strain. The possibility that this phenomenon was due to an alteration in the susceptibility of the cultures occurring during their serial propagation, rather than to a change in infectivity of the viruses, was investigated by re-titration of the original seed pools in both types of culture. The results of these titrations are shown in Table I. The only suggestion of a change in cell susceptibility is the lower titre of Lansing virus in the HeLa cell cultures of the later titration, but this difference is barely significant. Except for this anomalous figure the infectivity of each of the original pools was practically the same for both

TABLE II.—*Infectivity for HeLa and ERK Cell Cultures of Poliomyelitis Viruses Propagated by Serial Passage in ERK Cells*

		Brunhilde		Lansing		Leon	
		HeLa	ERK	HeLa	ERK	HeLa	ERK
1st passage	T	4·7	5·5	3·5	3·5	4·9	4·7
	D	—	6·3	—	4·3	—	5·3
2nd „	T	>4·1	>4·5	1·1	3·5	3·5	>4·5
	D	—	11·6	—	9·6	—	10·6
3rd „	T	3·7	3·9	1·5	2·9	2·4	4·7
	D	—	16·9	—	13·9	—	16·9
4th „	T	4·7	4·5	1·5	3·7	3·7	5·1
	D	—	23·2	—	19·2	—	23·2
Preparation of pools	D	—	25·2	—	21·2	—	25·2
Titration of pools	T	5·5	6·1	2·5	5·7	4·5	5·6
	D	—	31·2	—	27·2	—	31·2

T = Virus titre. D = Log_{10} of the reciprocal of the dilution of the seed pools used to initiate the series.

HeLa and ERK cell cultures and approximated closely to that observed in the earlier titration. The disproportionate virulence for HeLa and ERK cell cultures shown by the Lansing and Leon strains after passage in ERK cells appears, therefore, to be due to an adaptive change in the viruses rather than to an alteration in cell susceptibility.

The progressive dilution of the original virus inocula during serial passage was of the order of 10^{-5} at each titration. The dilution attained in each ERK cell culture used for passage is recorded in Table II where the figures for the final pools exclude any possibility of infection of cultures by carry-over of the original seed. The experiments thus provide conclusive proof of the growth of each type of virus to high titre in ERK cells throughout a series of subcultures. The identity and purity of the final virus products were established by neutralization tests using standard specific monkey antisera of the three serotypes. Doses of 100 TCD_{50} of each pool virus as measured by titration in ERK cells were neutralized by homologous but not by heterologous antisera when tested for cytopathogenic effect in ERK cell cultures.

DISCUSSION

The propagation of the three poliomyelitis virus prototypes in a cell strain derived from kidney tissue of a rabbit embryo supports Kret's claim that the tissue culture range of the Brunhilde, Lansing and Leon strains may be extended to cells obtained from animals other than primates. Both practical and theoretical aspects of this phenomenon appear to be of some importance. Among the former is the facility with which almost unlimited supplies of susceptible tissue cultures may be prepared without the use of monkeys or human serum. As can be seen in Table II such cultures are capable of producing high titre virus pools of each serotype suitable for use as antigens in poliomyelitis serological tests. Furthermore the production of poliomyelitis antisera in rabbits by the inoculation of virus grown in ERK cells maintained in rabbit serum medium appears to offer an elegant technique for the preparation of highly specific reagents. Whether or not the immunological uses of virus grown in ERK cells might be extended to the immunization of humans must depend on further work and theoretical considerations, the ease of cultivation of this cell line in either rabbit or bovine serum media being offset by the presence of these substances in the infected tissue culture fluids.

From the theoretical aspect the adaptation of poliomyelitis viruses to growth in cells derived from embryos of animals whose tissues are non-susceptible in primary explant (Barski *et al.*, 1954; Wood, personal communication) deserves some consideration. Three possible factors, tissue culture environment (Kaplan, 1955) embryonic origin (Wilson and Miles, 1955) and " transformation " (Westwood *et al.*, 1957) may singly or in combination be responsible for this phenomenon. It seems reasonable to suppose that there exists some fundamental difference of origin or metabolism between the cells of primary explants of rabbit kidney and the " transformed " cells of strain ERK. The investigation of such a difference might well throw light on the problem of virus adaptation.

SUMMARY

The three prototype poliomyelitis viruses can be propagated in a strain of cells (ERK) derived from kidney tissue of a rabbit embryo. Such cells provide

a satisfactory host system for the titration and production of these three viruses. The advantages gained by the use of the ERK cell strain are discussed together with its possible uses as a host system for bulk virus production. Some reasons for the susceptibility of cell strain ERK to poliomyelitis viruses are considered.

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HISTOCHEMICAL DEMONSTRATION OF THE EFFECT OF NERVE SECTION ON CHOLINESTERASE ACTIVITY AT MOTOR END PLATES IN THE GASTROCNEMIUS MUSCLE OF THE GUINEA-PIG

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THE present experiment was undertaken as a consequence of a recent paper by Snell and McIntyre (1956) in which they reported that no cholinesterase (ChE) activity was demonstrable histochemically in the motor end plates of guinea-pig gastrocnemius muscles 45 days after denervation. Their technique for demonstrating ChE activity was that of Koelle and Friedenwald (1949) and modified by Couteaux (1951) and Coërs (1953). These results are in conflict with our data on denervated cat gastrocnemius muscles (Groblewski, McNamara and Wills, 1956): in two experiments not more than about one-half diminution of ChE activity in the subneural apparatus was observed even at 54 and 56 days after denervation. Two possible explanations for this discrepancy in results were apparent: (1) a species difference or (2) an inhibitory effect of the formol-saline prefixation on ChE activity. Therefore in the present experiment a series of guinea-pigs was used and Gomori's simplification of Koelle's (1951) histochemical technique followed for testing ChE activity.

MATERIALS AND METHODS

Six male guinea-pigs, weighing 450–500 g., were lightly anaesthetized with nembutal. A 10-mm. section of the sciatic nerve was removed from the mid-thigh of the left leg. A few crystals of sodium sulphacetamide were inserted before closure of the incision, and about 20,000 units/kg. of penicillin G (Lilly) were injected intramuscularly into the right leg shortly after the operation. One animal each was killed with nembutal at 31, 38, 45, 52, 59 and 66 days after nerve section. The denervated gastrocnemius was removed from the left leg and the normal (control) gastrocnemius from the right leg. They were kept in a refrigerator for not more than 1 hr. until samples could be tested for ChE activity. From the 52-day animal, one half of each muscle (denervated and control) was placed immediately after removal from the animal in 10 per cent neutral buffered formalin. After 6 hr. of fixation, the tissue was washed overnight in running tap water. Next morning the tissues were drained and placed in a bottle in a refrigerator (about 5°) until the ChE test was performed.

Fresh muscle was teased in saline. Formalin-fixed muscle was teased in distilled water. Samples of teased muscle were incubated for 15 min. at 37° in syracuse dishes containing acetylthiocholine as substrate in the incubation solution. The latter was made up according to Gomori's simplification of Koelle's (1951) technique. Always an aliquot of this solution was divided into two equal portions, one for the denervated muscle sample, the other for the control sample, and the two portions were incubated more or less simultaneously. Several pieces of the teased muscle were then either mounted in glycerine on a slide and the coverslip sealed with mastic-paraffin or dehydrated through the alcohols, cleared in toluene and mounted on slides with Permount. The subneural apparatus of the motor end plate,

the site of ChE activity, was made visible by the copper sulphide precipitate. The amount of ChE activity was assessed by the amount of precipitate. The latter always appears lighter per unit area when the subneural apparatus is expanded than when it is contracted.

RESULTS

Gross Appearance of Denervated Gastrocnemius Muscle

At 31 days after denervation, the delicate muscle fibres were obviously thinner than in the control muscle. At 38 days the whole muscle was obviously smaller than the control; the fibres were very thin and delicate and there was much connective tissue. At 45 days there was much fat as well as connective tissue occupying the space normally filled by the gastrocnemius muscle. At 52 days the outer bundles of fibres were almost entirely masses of fat and connective tissue. However, the fibres of the bundles closer to the tibia were merely thin and delicate. At 59 days there were relatively few muscle fibres; they were very thin and there was much fat and connective tissue. At this time and at 66 days, the gastrocnemius was only about one-fourth the size of the corresponding control muscle. Actually the 59-day animal showed the worst degeneration but this may be an instance of individual variation.

Microscopic Appearance of Denervated Muscle

Fresh tissue.—At 31 days the ChE activity was somewhat less than in the control muscle but the more obvious difference between them was in the size of the subneural apparatus (Fig. 1 and 2). In the denervated muscle most of them were small and compact. The pattern of the synaptic groove could not be seen. Expanded ones were paler and more or less fragmented. The muscle fibres were narrower than in the control sample. At 38 days ChE activity was about one-half that of the control. The subneural apparatus was smaller and thinner. Details of structure usually could not be made out. Fat cells were present throughout the muscle fibres. At 45 days and later, in fibres from the more atrophied bundles, the ChE activity was only about one-quarter as much as in the control; in the thin fibres closer to the tibia it was about one-half. Also in the more atrophied bundles there were not only the usual bands of small motor end plates but also many scattered ones, weaker in intensity, so that the total number of motor end plates seemed to be greater. In them the pattern of the subneural apparatus usually was poorly defined or lost entirely, the ChE activity being indicated merely by a blob of precipitate. Many of these fibres were so degenerated that the bands of motor end plates were only faintly visible in the clear glycerine mounts of the teased tissue. They are difficult to demonstrate in photographs because of the thickness of the pieces of teased muscle. Fig. 3–5 show the contrast between fibres from control and denervated legs of an animal 52 days after denervation; Fig. 6–8 show the same in an animal 66 days after denervation. In the controls (Fig. 3 and 6) the muscle fibres are wide, the subneural apparatus large and the pattern clearly defined. Figs. 4 and 7 illustrate the compact kind of subneural apparatus found in denervated muscle. In Fig. 5 and 8 some of the motor end plate loci are clearly defined, others not.

Formalin-fixed tissue.—These samples were taken from an animal 52 days after denervation. The ChE activity in the formalin-fixed control muscle was about one-half as much as in the fresh muscle (compare Fig. 3 with Fig. 9). In

the sample from the denervated leg the ChE activity was discernible only in the small compact motor end plates (Fig. 10, 11). The very degenerated subneural apparatus described above in the fresh teased muscle could not be seen at all. It was evident that fixation by formalin had reduced the ChE activity by about one-half.

DISCUSSION

Snell and McIntyre (1956) discuss the confusing results which have been reported from manometric determinations of ChE activity in denervated muscle. Some of the contradictory results followed calculation of enzymic activity per 100 mg. of muscle or per weight of the whole muscle. It is not necessary to review this literature. It may be permissible, however, to note that the particular muscle used may make a difference : Brooks and Myers (1952) reported different results for the cranial serratus muscle from those for the gastrocnemius muscle of the guinea-pig. There may also be species differences in the rate at which atrophy occurs after denervation. In our experience it seemed to be more marked in the guinea-pig than in the cat after the 45th day.

The purpose of this paper, however, is to offer a warning. If the results obtained by the manometric method may be invalid because adequate precautions were not taken, the same is true of a histochemical technique for demonstrating ChE activity. Seligman, Chauncy and Nachlas (1951) showed that esterase activity in rat liver was reduced one-half after 2 hours' fixation in 10 per cent formalin at 25°. Couteaux (1951) and Taxi (1952) reported a loss of ChE activity after formalin fixation but found that there was less loss in striated muscle than in some other tissues. The failure of Snell and McIntyre to demonstrate ChE activity histochemically 45 days after nerve section in the gastrocnemius muscle of the guinea-pig seems to have been mainly a consequence of formalin prefixation of the tissue. In fresh tissue, ChE activity could be demonstrated, even at 66 days, the termination of the experiment.

EXPLANATION OF PLATE

Photomicrographs of portions of guinea-pig gastrocnemius muscles. Subneural apparatus of the motor end plates is stained by the Koelle histochemical technique. × 135.

FIG. 1.—Fresh normal gastrocnemius muscle from right leg of guinea-pig 31 days after nerve section in left leg.

FIG. 2.—Fresh denervated gastrocnemius from left leg of the same animal. Muscle fibres narrower, subneural apparatus compact and smaller than in the normal leg.

FIG. 3.—Fresh normal gastrocnemius from right leg of guinea-pig 52 days after nerve section in the left leg.

FIG. 4.—Fresh denervated gastrocnemius from left leg of the same animal. Subneural apparatus is small, compact and narrow.

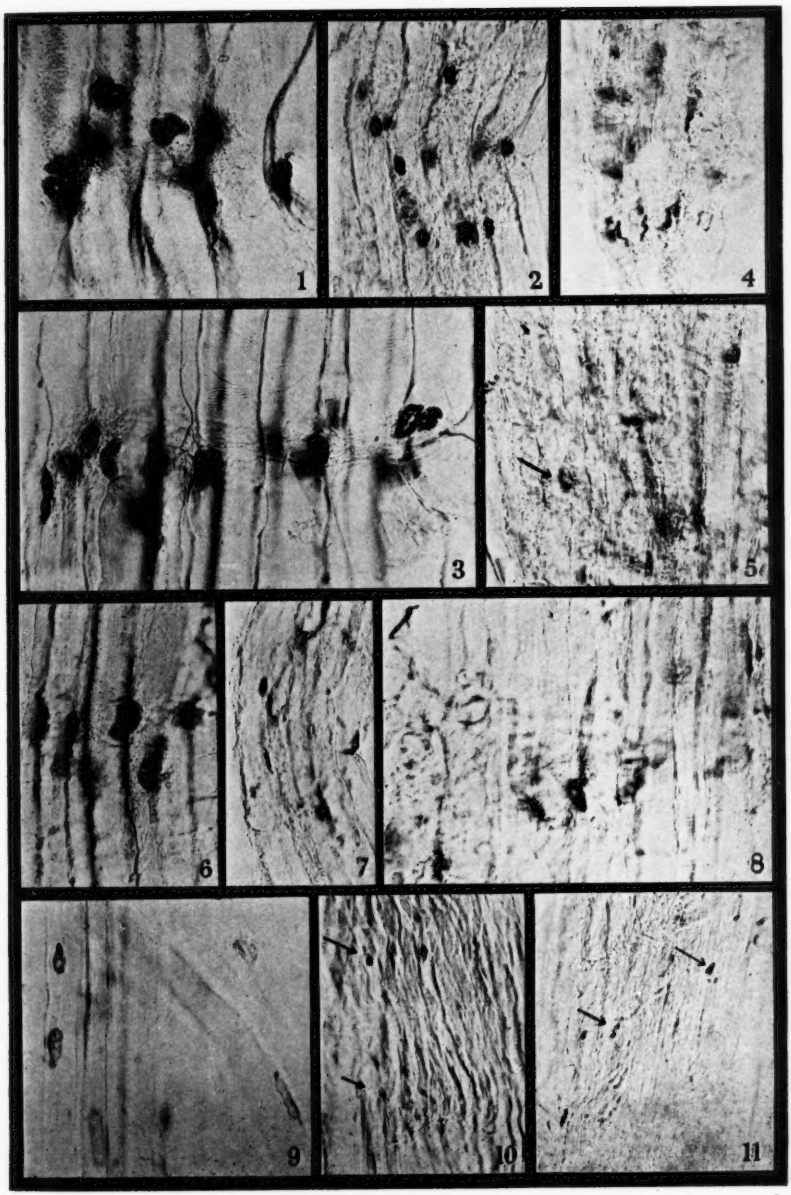
FIG. 5.—Same. Pattern of the subneural apparatus usually is poorly defined or fragmented. Arrow points to one of them.

FIG. 6.—Fresh normal gastrocnemius from right leg of guinea-pig 66 days after nerve section in the left leg.

FIG. 7 and 8.—Fresh denervated gastrocnemius from the left leg of the same animal. Subneural apparatus may be small, narrow and compact or vaguely defined.

FIG. 9.—Formalin-fixed normal gastrocnemius from right leg of guinea-pig 52 days after nerve section in the left leg. Same animal as for Fig. 3. Cholinesterase activity decreased about one-half by formalin.

FIG. 10 and 11.—Formalin-fixed denervated gastrocnemius from the left leg of the same animal. Subneural apparatus is visible when it is small and compact. Arrows point to a few of them.



Bergner.

Judging by the intensity of the precipitate, it may be concluded that in our experiment activity was reduced to a fourth or less in the pale dispersed or disintegrated motor end plates but to only about one-half in the small compact ones. The chief characteristic was the small size of this area. This reduction in size, as shown by Kupfer (1951), parallels the decrease in cross-sectional area of muscle fibre after denervation. Our results are in keeping with those of Stoerk and Morpeth (1944) who showed that ChE activity in the rat was reduced to about one-third shortly after denervation but that the reduction was not a function of time. It was the same at 21 as at 2 days.

Since some ChE activity was demonstrated in formalin-fixed gastrocnemius muscle 52 days after denervation (Fig. 10, 11) whereas Snell and McIntyre found none at 45 days, the sensitivity of this histochemical technique seems to vary somewhat with the modifications to the technique. Harris, Cohen and Bergner (1953) correlated Koelle's (1951) technique with Ammon's quantitative method in order to estimate the threshold of activity per unit area which is necessary to result in a visible precipitate of copper sulphide. Gomori's variant of it seems to be just as sensitive.

SUMMARY

The Koelle histochemical technique for demonstrating cholinesterase activity was used on denervated gastrocnemius muscle of the guinea-pig.

When fresh teased muscle was used, ChE activity at motor end plates in denervated muscle was reduced to from one-half to one-fourth that of the contralateral control muscle by 45 days and there was no further marked reduction even at 66 days.

Prefixation with formalin partially inhibited ChE activity. Consequently, in formalin-fixed denervated muscle this reduction in ChE activity was much greater than in fresh muscle.

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THE INTRATHECAL TUBERCULIN REACTION IN THE RABBIT

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THE response of the sensitized meninges to intrathecal tuberculo-protein has been studied in man (Smith and Vollum, 1950; Swithinbank, Smith and Vollum, 1953) and in the guinea-pig (Burn and Finley, 1932; Bosanquet, Daniel and Vollum, 1953). There are limitations to the observations which can be made in man, and in the guinea-pig reactions of the meninges can only be studied histologically, since it appears to be impossible to obtain by cisternal puncture samples of cerebro-spinal fluid (C.S.F.) consistently blood-free and sufficient for cell and protein determination.

The meningeal response to tuberculin has therefore been studied in the rabbit, from which animal C.S.F. may be obtained by cisternal puncture in amounts of approximately 1 ml. This animal does not sensitize well to tuberculin, as judged by the skin reaction, and negative findings have been reported previously (Bickford, 1932). The plan of the experiment was as follows. Rabbits known to be skin sensitive to tuberculo-protein (Weybridge human purified protein derivative, P.P.D.) were given small quantities of P.P.D. into the cisterna magna, the subsequent response of the meninges being followed by observations of the C.S.F.

Since changes in the normal distribution of bromide between serum and C.S.F. have been noted in human subjects, especially during the course of a meningitis (Walter, 1929; Taylor, Smith and Hunter, 1954), similar studies have been made during the course of a meningeal reaction in these rabbits.

METHODS

Rabbits of mixed domestic strains and approximately equal weight (*ca.* 1750 g.) were used.

Sensitization.—One antigen was used consistently throughout, a live murine tubercle bacillus (Vole OV). Each animal received 1 ml. of a heavy suspension in saline of this organism into the groin. The dose was not calibrated, since adequate sensitization was the only object. In only one animal was more than one injection required to achieve this. Surviving animals, used for repeat and control experiments, received a second booster dose of vole bacilli, because their skin sensitivity tends to wane after some weeks.

Skin sensitivity.—Intradermal injections of 1·5 μg. of P.P.D. in 0·2 ml. saline were given at weekly intervals after inoculation with the vole bacillus. Skin tests were read after 24 and 48 hr. An area of erythema with an indurated centre of oedema was taken as positive and these animals are termed sensitized.

The meningeal reaction.—P.P.D. in doses of 1·5 μg. in 0·2 ml. isotonic saline was injected intracisternally, by replacement of C.S.F., to avoid sudden increases in pressure and to obtain a baseline sample of C.S.F. for examination. The animals were lightly anaesthetized with intravenous nembutal and their heads held fully flexed by an assistant; this was found more satisfactory than any form of head clamp. At intervals from 8–216 hr. after the challenging dose, C.S.F. was removed from the same site in the same way.

* While in receipt of a personal grant from the Medical Research Council.

About 0·6–1·0 ml. of macroscopically blood-free C.S.F. may be obtained in this way, in our experience, not more frequently than every 16 hr.

Cells were counted always within 6 hr. of sampling, using $\frac{1}{2}$ dilution of C.S.F. with toluidine blue (0·25 per cent) in a Fuchs-Rosenthal chamber. C.S.F. protein was estimated turbidimetrically, using a photometer adapted for this purpose.

During the course of the meningeal reaction, studies of the changes of concentration of bromide in the C.S.F. following I.V. injection of sodium bromide were made. The bromide was given as a 13·3 per cent solution in water into an ear vein, the dose usually being 2 ml. (266 mg. NaBr). Blood was obtained either from an ear vein or by cardiac puncture and both serum and C.S.F. were collected and stored in bromide-free glass tubes. The bromide in these fluids was estimated by the method of Hunter (1955) and chloride by the method of Sanderson (1952).

Controls.

(*a*) Non-sensitized animals, with negative skin tests, were given intracisternal P.P.D., and specific tuberculo-polysaccharide (kindly supplied to us by Dr. Philpot of the Department of Pharmacology, Oxford), by replacement of C.S.F., in equal volume. C.S.F. samples were taken subsequently for examination. Two control animals also received tuberculo-polysaccharide intradermally, for comparison with the effects of the same injections in sensitized animals.

(*b*) Sensitized animals received isotonic saline, diphtheria antitoxin and tuberculo-polysaccharide intracisternally, the C.S.F. responses being followed in the same way as those following P.P.D. injections in the same animals. Two sensitized animals were injected with large doses of P.P.D. (37·5 μg.) intraperitoneally and two received intradermal tuberculo-polysaccharide.

RESULTS

The degree of skin sensitization in the rabbits inoculated with the vole bacillus is variable. Of the first 12 animals only 1, however, was negative on skin testing 30 days later and a second inoculation in this animal was followed by the development of sensitivity.

Both sensitized and control animals tolerated the intracisternal injections well and it was never possible to determine which animals were undergoing a meningeal reaction except from the C.S.F. findings.

Changes in the C.S.F.

The cell and protein changes in the C.S.F. in response to intrathecal P.P.D. in the non-sensitized animals are shown in Table I; those in the sensitized animals in Table II. An indication of the degree of skin sensitivity in the latter at the time of the intrathecal tests is given in Table II.

In Table III are shown the C.S.F. changes following the intracisternal injection of substances other than P.P.D. in sensitized and non-sensitized animals.

Table IV shows the difference between serum and C.S.F. bromide concentration in normal rabbits at a time after I.V. injection when the relationship is essentially constant, and also in sensitized rabbits.

There is a significant change in this "steady-state" relationship at the peak of an intrathecal reaction (24–48 hr. after P.P.D. injection) but by 168 hr. a return to the normal has occurred.

The rate of increase in concentration of bromide in the lumbar C.S.F. following I.V. bromide injections has been studied in patients with no disease of the C.N.S. and in those with meningitis, especially tuberculous meningitis (Bourdillon, Fischer-Williams, Smith and Taylor, 1957). The rate of increase is higher in

meningitis than in the normal and there is a relationship between this rate and the "steady-state" value for bromide. Values for the cisternal C.S.F.-serum bromide ratio 2, 4, 6 and 8 hr. after I.V. bromide in the rabbit are shown in Table V and the values obtained during the course of a P.P.D. reaction and after the intrathecal injection of substances producing no detectable meningeal response are shown in Table VI.

TABLE I.—*Effect of Intracisternal P.P.D. on C.S.F. Protein and Cells in Normal Rabbits*

Rabbit No.	Hr. after P.P.D.	Protein (mg./100 ml.)	Cells per cu. mm.		
			R.B.C.	Poly.	Mono.
A	16	18	10	0	2
B	16	20	0	2	2
C	16	20	60	0	4
D	16	17	4	0	0
E	8	32	100	2	8
H	8	11	8	0	4
18	24	20	84	4	8
	48	82	40	12	24
	96	40	4000	2	4
	144	10	10	0	0
	192	0	60	0	8
19	24	19	12	0	2
	48	22	650	0	12
	96	19	200	0	2
	144	43	6000	0	0
21	24	19	6	0	4
	48	14	180	22	6
	96	15	40	2	0
	144	12	2	2	0
23	24	35	2	0	2
	48	135	9000	6	6
	96	25	76	2	2
	144	20	6	2	0
	192	—	10	0	4

— = No determination.

DISCUSSION

The reaction to intrathecal P.P.D. in the sensitized animals appears to be of a different order from that in the controls and seems to be a manifestation of a specific reaction of the meninges. This response does appear to conform to a pattern in which are seen a rise in the C.S.F. protein content and a marked pleocytosis, these changes being maximal 8–24 hours after cisternal injection. Smears made of the spun deposits of these fluids have shown that the cells are mainly of two types; polymorphs (pseudo-eosinophils) and mononuclears. A few true eosinophils are also seen. Of the mononuclears, the majority have the appearance of lymphocytes but a number of larger possibly macrophage cells are also present. The initial spike of cells is predominantly polymorph in most instances, although in one animal (No. 11) there were twice as many mononuclears as polymorphs at 8 hours, the usual pattern being seen 16 hours later. In every case the mononuclear increase in the C.S.F. persists longer than does the polymorphonuclear. Swithinbank *et al.* (1953) reported that in human subjects with naturally acquired tuberculin sensitivity, a clear-cut diphasic pleocytosis with

TABLE II.—*Effect of Intracisternal Injection of P.P.D. in Tuberculin-sensitized Rabbits*

Rabbit No.	Skin sensitivity	Hr. after injection	Protein (mg./100 ml.)	Cells per cu. mm. R.B.C.	Poly.	Mono.
6	++	24	40	490	294	50
		48	42	8,000	190	60
		96	68	400	20	130
		216	40	400	0	75
7	+++	0	25	2	0	0
		8	60	20	238	96
		24	55	84	562	20
		48	155	12,800	42	10
		72	60	180	80	50
		96	50	170	40	45
		120	34	55	20	70
		144	20	105	10	100
		216	25	0	0	35
11	+	0	21	—	—	0
		8	200	2,640	222	420
		24	300	8,800	1,080	50
		48	220	554	74	18
		72	160	8,000	10	30
		96	—	1,600	10	70
12	++	0	17	622	0	2
		8	150	14,000	190	90
		24	105	8,000	1,480	200
		48	115	3,200	240	124
		72	55	1,600	100	110
		96	50	800	10	20
		216	34	650	0	130
15	—	0	17	2	0	0
		48	55	1,440	100	20
17	+++	8	—	200	1,060	450
		24	95	64	430	64
		96	—	12,000	50	200
		216	17	130	10	70
19	+	0	35	0	0	2
		8	41	0	56	36
		24	40	146	282	42
		48	37	46	190	138
		72	120	400	120	180
		96	70	85	65	105
		120	34	85	60	145
		144	45	100	5	160
		216	28	345	0	30
20	+++	0	22	0	0	2
		24	45	9,600	800	40
		48	60	2,900	70	40
		72	25	936	150	190
		96	—	11,200	25	75
		144	40	2,400	10	60
		168	—	4,000	10	20
21	+++	0	17	—	—	—
		16	45	100	844	88
		40	47	800	280	20
		120	25	800	280	20
		144	15	230	20	130
		168	30	35	0	55
		192	25	48	0	32
		216	25	0	0	12
22	+	0	13	—	—	—
		16	31	160	1,418	36
		120	25	200	10	90
		192	21	10	5	100
		216	25	2,500	5	70
23	+++	0	13	—	—	—
		16	85	4,000	738	48
		40	125	1,220	120	60
		120	20	20	0	40
		144	25	30	0	40
		168	37	1,420	2	38

— = No determination.

TABLE III.—*Effect of Intracisternal Injection of Substances other than P.P.D.*

Rabbit No.	Skin sensitivity	Substance injected	Hr. after injection	Protein (mg./100 ml.)	Cells per cu. mm. R.B.C.	Poly.	Mono.
11	+	0·2 ml. isotonic saline	0	30	0	0	2
			24	35	180	20	90
19	+	80 mμg. specific polysaccharide in 0·2 ml. saline	0	40	0	0	0
			24	30	400	40	70
			48	—	910	0	0
			72	41	0	0	10
25	—	Ditto	0	20	30	0	4
			24	—	900	20	60
			48	—	600	80	50
			72	100	800	0	0
6	+	1 mg. specific polysaccharide in 0·2 ml. saline	0	—	54	0	2
			24	—	1520	1010	30
26	—	Ditto	0	—	0	0	0
			24	—	190	1490	5
19	+	400 units diphtheria antitoxin (0·2 ml.)	0	—	100	0	0
			24	—	20	160	20
6	+	Ditto	24	77	960	0	20
			48	—	9200	10	30

— = No determination.

TABLE IV.—*Bromide Ratio (C.S.F./Serum Concentration) after I.V. Injection of 266 mg. NaBr in Normal Rabbits and after Intrathecal P.P.D. Injection in Sensitized Rabbits*

Number of animals	Time after P.P.D. (hr.)	Bromide ratio Mean	Bromide ratio Range	Chloride ratio Mean	Chloride ratio Range	Protein (mg./100 ml.) Mean	Protein (mg./100 ml.) Range
12	No P.P.D.	0·90	0·76–1·05 (15 observations)	1·35	1·27–1·44 (5 observations)	28	15–55
5	24–48	1·07	1·00–1·13	—		115	75–138
5	168	0·89	0·75–1·02	—		60	25–92 (4 rabbits)

TABLE V.—*Bromide Ratio after Intravenous Injection of NaBr in Normal Rabbits*

Number of animals	Time after Br injection (hr.)	Bromide ratio Mean	Bromide ratio Range
3	2	0·44	0·43–0·44
5	4	0·63	0·57–0·67
5	6	0·65	0·54–0·72
3	8	0·75	0·71–0·78

TABLE VI.—*Bromide Ratio 6 Hours after Intravenous Bromide Injection and 24 Hours after Intracisternal Injection of Various Substances*

Rabbit No.	Tuberculin sensitivity	Substance injected	Bromide ratio	Protein (mg./100 ml.)
11	+	0·2 ml. isotonic saline	0·81	35
6	+	400 units diphtheria antitoxin in 0·2 ml. isotonic saline	0·86	77
15	—	1·5 μg. P.P.D. in 0·2 ml. isotonic saline	0·92	60
15	+	Ditto	1·03	50
6	+	,,	0·90	75
11	+	,,	1·02	90
12	+	,,	1·13	—
17	+	,,	1·05	120
20	+	,,	0·92	63
24	+	,,	1·04	150
19	+	80 mμg. tuberculo-polysaccharide in 0·2 ml. isotonic saline	6·80	30
25	—	Ditto	0·89	—

double protein spike is seen in the C.S.F. after lumbar intrathecal P.P.D. injections. In recently vaccinated individuals, previously Mantoux-negative but becoming positive as a result of vaccination, the meningeal reaction was closely akin to that seen in the rabbit, in that only the first part of the double response was observed. However, in one animal (No. 19) at 144 hours a second rise in C.S.F. protein associated with a small mononuclear spike was seen and this could not be accounted for by a preceding traumatic haemorrhage, which after a number of cisternal punctures is difficult to avoid; this is more nearly like the results in the naturally sensitized human subject than any others we have obtained.

Specificity of the meningeal reaction

Reports that the response to non-specific foreign substances is more marked in tuberculous than in non-tuberculous animals (Dienes and Mallory, 1932) suggested that the meningeal response to such substances should be studied. The limited observations tabulated here do not confirm the existence of such a phenomenon, our findings being similar to those of Bosanquet *et al.* (1953) in the guinea-pig.

Our results suggest, too, that the meningitic response is to tuberculo-protein and not to tuberculo-polysaccharide. P.P.D. contains, at the most, 5 per cent by weight of polysaccharide. Injection of 80 mμg. of specific tuberculo-polysaccharide (the expected amount in 1·5 μg. of P.P.D.) in 0·2 ml. of saline produced no rise in C.S.F. protein and no more rise in cell content than following saline injection alone. A very large dose of the polysaccharide (1 mg. in 0·2 ml. saline) produced a very marked polymorphonuclear rise in both a sensitive and non-sensitive animal at 24 hours, which could unfortunately not be followed because of difficulties in puncturing both of these animals.

It is relevant to mention here that the intradermal injection of 80 mμg. specific tuberculo-polysaccharide in a control and a sensitized animal produced no macroscopic reaction. A very large dose of 1 mg. produced an irregularly defined erythematous area about 4 cm. in diameter, developing fully over 24 hours and disappearing as rapidly. The extent of this response was the same in both the control and the sensitized animal. The reaction was quite unlike the skin reaction to P.P.D. in the sensitized animal, both in its duration and absence of oedema.

Correlation of the sensitivity of the skin and meninges

It is reasonable to suppose that the severity of the meningeal reaction to P.P.D. will depend on the sensitivity of the meninges to tuberculo-protein, and previous work in the human subject has suggested that following vaccination with the vole bacillus of the Mantoux-negative individual the skin and the meninges both become tuberculin-sensitive at about the same time (Swithinbank *et al.*, 1953). In patients with tuberculous meningitis the size of the Mantoux reaction is a useful guide to the dose of intrathecal P.P.D. which has to be given to produce a meningitic reaction. In the rabbit no such correlation has been apparent. Thus in animals No. 11 and 22 (Table II) the pleocytosis following an injection of P.P.D. intrathecally was similar to that in animals No. 17, 20 and 23, which showed a markedly greater skin sensitivity. The range of sensitivity in the human subject is much greater than that in the rabbit and this is the most likely explanation of our findings. It is unfortunate that rabbit No. 15,

which, although vaccinated, was Mantoux-negative at the time of the cisternal injection, yielded samples of C.S.F. too contaminated or insufficient for examination. In this animal, the pleocytosis at 48 hours may have been due to previous trauma and bleeding and not mark a phase in an active meningeal response to P.P.D.

The response of the meninges to intraperitoneal P.P.D.

No abnormalities were found in the cisternal C.S.F. obtained from 2 sensitized rabbits 24 hours after receiving intraperitoneal injections of 37·5 μg. P.P.D. in saline. Typical of the minor response of the sensitized rabbit to tuberculin is the failure of either of these 2 animals to show any upset whatever, whereas Bosanquet *et al.* (1953) were able to produce death in sensitized guinea-pigs so treated.

The bromide distribution between serum and C.S.F.

Various explanations for the plasma-C.S.F. bromide difference in the normal may be proposed. Expressed simply, it must be due to active exclusion or excretion of bromide from the C.S.F. or both, unless some sort of bromide detoxication by the C.N.S., as proposed by Davson (1956) can be held responsible; but a conversion of bromide to bromate, as he suggests, seems unlikely. Why such disturbances of this ratio should occur so markedly during the course of a bacterial meningitis is not known but it has been postulated, since the same change is seen following the intrathecal injection of P.P.D. into the sensitized patient, that it is a manifestation of an antigen-antibody reaction occurring within the leptomeninges (Smith, Taylor and Hunter, 1955). Such reactions may inhibit active exclusion or excretion of bromide. The present study was an exploratory one, an attempt to elucidate such problems.

Unfortunately the differences between serum and C.S.F. bromide concentrations, at a time after I.V. injection when the relationship is essentially constant, is much smaller in the rabbit than in the man. The mean in the normal human subject (C.S.F./serum) is 0·38 (Taylor *et al.*, 1954). In the rabbit the mean of 15 determinations (Table IV) is 0·90 $\pm$ 0·02 (S.E.), values much closer to those found for chloride than in man, but still showing somewhat less bromide in the C.S.F. than in the serum. Davson (1955), in an unstated number of rabbits, found a ratio of 0·715 $\pm$ 0·02 using radio-bromide. A disturbance of this "equilibrium" does occur during an intrathecal tuberculin reaction in the rabbit, but the change is a small one. Thus at 24 hours from the outset of a meningeal response there was noted a rise in the C.S.F./serum bromide ratio, above unity and approaching that for chloride, such as is found in tuberculous meningitis (Taylor *et al.*, 1954). The return to a normal "steady-state" ratio is much quicker than in the human subject (Swithinbank *et al.*, 1953).

We have concluded from this that the mechanisms responsible for maintaining the C.S.F./serum bromide concentration differences in the human subject are functionally better developed than in the rabbit and that depression of the activity of these mechanisms is not to be so convincingly demonstrated in the rabbit as in man.

By measuring, instead, the bromide concentration in the C.S.F. before a "steady-state" is reached, it is possible to show a greater difference between the normal and the meningitic animal. However, there are certain disadvantages

inherent in this method. Thus, these "rate of entry" tests cannot be repeated in the same animal, since bromide is retained for long periods. We have attempted to overcome this difficulty by using radio-active bromide (^{82}Br) and it appears that in this way repeated tests may be possible.

A second disadvantage is apparent from a study of the results obtained when substances producing little or no meningeal response (judged by the cell and protein contents of the C.S.F.) have been injected intracisternally. These lie midway between the normal and meningeal results and suggest that tampering with a C.S.F. system as small as that of the rabbit may produce upsets *per se*. More data are needed to test this matter.

It does seem that the study of various types of experimental intrathecal reactions may be possible in the rabbit and we consider that bromide and possibly other substances may be of value in such studies, to elucidate the nature of these reactions and the means by which many substances, of which bromide is one, are partially or wholly excluded from the C.S.F. and C.N.S.

SUMMARY

A specific sensitivity of the leptomeninges of the rabbit to tuberculo-protein has been produced and the meningitis resulting from the injection of this protein intracisternally has been studied in serial samples of C.S.F.

During the course of these reactions changes have been noted in the normal distribution of bromide between C.S.F and serum. At equilibrium these changes are slight, probably because the initial differences in concentration in the two fluids are small. Before equilibrium is established, however, the differences are much greater and may provide a measure of the degree of meningeal response.

The rabbit appears to be a useful animal for the study of meningeal reactions.

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A METHOD FOR THE STUDY OF IMMUNITY TO TUBERCULOSIS

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IT has been observed that when mice are inoculated intracorneally with *Mycobacterium tuberculosis* (bovine strain) the lesions reach a maximum at about 30 days after inoculation and then gradually decrease (Rees and Robson, 1950). This suggests that an immunity process has set in which protects the eye against the further spread of the disease. This is, in fact, in keeping with the ideas advanced by Bloch (1954) based on experiments in which he inoculated mice intravenously with various doses of organisms and found that whereas with large doses the great majority of animals died after about 20 days, with smaller doses the great majority of animals remained alive, though macroscopic and microscopic lesions regularly occurred. He attributed the absence of mortality in the animals receiving smaller doses to the fact that immunity developed during the period when the organisms were multiplying.

In the present experiments, therefore, one eye was inoculated with *Myco. tuberculosis* and when the lesion had reached a maximum and was no longer progressing, the second eye was inoculated in order to determine whether an immunity process had set in. It was found that such second eyes did not develop any macroscopic lesions (as already reported by Robson and Didcock, 1956). A study was then made of the changes which occurred in these " immune " eyes in order to obtain information about the mechanism of immunity.

MATERIALS AND METHODS

Albino mice of the " C " strain were used throughout, aged 3–4 months and weighing 20–25 g. at the beginning of the experiment. They were bred in the animal house at Guy's Hospital Medical School.

Organisms used were :

1. A bovine strain of *Myco. tuberculosis* originally obtained from Dr. Levinthal.
2. B.C.G., Copenhagen strain.
3. H37Ra obtained direct from Dr. Steenken of the Trudeau Laboratories.

Cultures were maintained in Dubos medium and the virulent strain was passaged through mice from time to time. Suitable dilutions in normal saline containing 0·05 per cent Tween 80 were prepared just before use from 7–10 day cultures for the bovine strain and from 17–19 day cultures for the B.C.G. and H37Ra strains. For the bovine strain the culture was diluted either 1/100 or 1/1000 and the results obtained with these dilutions were essentially similar. Cultures of B.C.G. and H37Ra were used either undiluted or diluted 1/10 or 1/100. Where dead bacilli were used they were autoclaved for 20 min. at 120°.

The technique of intracorneal inoculation was that of Rees and Robson (1950). The immunizing dose was usually given intracorneally into one eye but in one experiment it was given intradermally into the skin of the ear or of the flank. The immunizing dose given intradermally was about 0·02 ml. of a 1/100 or 1/1000 dilution of the bovine strain and both dilutions gave the same results. The challenging dose was given intracorneally 28–30 days

later. The dilution of the immunizing and challenging dose of the bovine strain was always the same.

The eyes were examined by four techniques, each of which is capable of showing the presence of an active or quiescent infection, namely :

1. Examination by use of a binocular dissecting microscope (Rees and Robson, 1950).
2. Phase contrast examination of the intact excised cornea by use of the method of Robson and Didcock (1955).
3. Gross staining of the intact excised cornea (Robson and Sullivan, 1957).
4. Histological examination of sections of corneas. These were fixed in Bouin's solution, cut at 5 μ and stained either with haematoxylin and eosin or by the Ziehl-Neelsen method and counterstained with haematoxylin.

These techniques made it possible to build up a comprehensive picture of the course of an infection.

RESULTS

Macroscopic appearance

In a primary infection with a 1/100–1/1000 dilution of the culture of the bovine strain a macroscopic lesion appears after a latent period of about 7–12 days ; these lesions gradually increase in size to involve most of the cornea. When " immune " mice were challenged intracorneally, no macroscopic lesions developed over periods of observation of up to 7 months and the cornea remained perfectly clear. This applied to animals immunized with the bovine strain either by intracorneal or by intradermal injection. When mice were inoculated intracorneally with an undiluted culture killed by heat this failed completely to produce any immunity to a subsequent challenge of the second cornea with a 1/1000 dilution. The results with B.C.G. and H37Ra immunization are described below.

Microscopic appearance

When *Myco. tuberculosis* was injected into the cornea of mice previously immunized, the most striking appearance microscopically was the lesser cellular reaction throughout, as compared with the reaction to a primary infection. As a result, details of the centre of infection could be made out by the phase contrast technique at almost all the observations, whereas with a primary infection the centre usually becomes obscured by a solid core of cells within a day or two of inoculation.

On the 1st day after inoculation phase contrast examination showed leucocytes and macrophages over an area of about 200 μ in diameter around the needle track. Bacilli were found both intracellularly and extracellularly. Histologically, a mainly polymorph reaction, with some macrophages, was found at this stage.

By the 3rd day, it could be seen histologically that there was an increase in the percentage of mononuclear cells, and by the 5th day the reaction as seen both by phase examination and by histology had changed to a typical mononuclear picture with only a few polymorphs, mainly neutrophils, present.

For the next 12 days the appearance of the lesion remained unchanged and phase contrast examination showed that it consisted of a thin core of cells, mainly mononuclear, with no increase in density.

At 17 days, phase contrast showed groups of cells which, at a higher magnification, were seen to consist of a cell with a large pale oval nucleus, similar in appearance to the corneal corpuscles seen in the normal cornea, surrounded by a few leucocytes and macrophages. These distinctive aggregates of cells were found in all the immune corneas examined after this time and have been called

" corpuscular units ". Bacilli were found at this time within both corpuscles and macrophages. At the same time histological examination of stained sections showed invasion of the cornea by lymphocytes. We were, however, unable to identify in the sections, the " corpuscular units " as distinct entities. This may be due to the fact that, for phase contrast, the cornea is flattened out and viewed in the plane of the lamellae of the substantia propria. Since the invading cells in the cornea move in the layers between the lamellae, the spatial relationships of the cells is best appreciated when viewed in this manner. When cutting sections, however, due to the curvature of the cornea it is inevitable that the majority of sections will be cut transversely to the lamellae of the substantia. Thus, although the anteroposterior relationship of the cells is easily seen in sections the actual relationships of the cells at right angles to this, which are in the plane of the lamellae, and are probably more important, are not seen. The actual significance of the " corpuscular units " is still unknown.

Corneas examined over the succeeding 4 months showed that there was no change either in the phase contrast appearance or histologically. At the end of this period, however, both methods of examination suggested that the lesion was regressing somewhat, since the overall size and density of the cellular focus was decreased.

Erythrocytes, previously described as " grey cells " (Robson and Didcock, 1955) were also seen at the various stages described above. Microscopic vascularization was occasionally seen in the later stages in corneas macroscopically clear.

Fate of organisms

This was investigated quantitatively by staining the bacilli *in situ*. By this method the mass of bacilli can be seen at a magnification of 120 × in about 80 per cent of the corneas examined immediately after inoculation when the inoculum consists of a 1/100 dilution, *i.e.*, about 5000 organisms. Corneas of immune mice were inoculated with such a dilution and examined at intervals up to $4\frac{1}{2}$ months later. At no stage was there any evidence of multiplication and up to 3 months after inoculation the number of organisms appeared to remain constant ; they were always found to be intracellular, both by the gross staining technique and in sections. After $4\frac{1}{2}$ months, three corneas were examined by gross staining and no bacilli were found, suggesting a diminution in the number of bacilli. The organisms were morphologically normal up to 4 weeks after inoculation, but many of them appeared beaded at later stages, suggesting degenerative changes. However, a cornea transferred to Kirschner's medium 27 days after inoculation gave positive cultures. Another two corneas transferred 135 days after inoculation failed to give any growth even after 6 weeks in Kirschner's medium. When these were stained with carbol fuchsin, the number of organisms seen was approximately the same as that originally inoculated.

B.C.G. and H37Ra

It seemed of interest to determine whether inoculation with these strains of *Myco. tuberculosis* would produce immunity detectable by the method described and they were therefore injected into one cornea. The immunizing dose of H37Ra was 0·01 ml. of an undiluted culture, whereas with B.C.G. the culture was diluted 1/100. This corresponded approximately to 100,000 H37Ra organisms per

injection and 200 B.C.G. organisms. No macroscopic lesions developed in any of the eyes receiving the immunizing dose, these being observed up to 7 months after B.C.G. and up to 4 months after H37Ra.

A month later the second eye was challenged with a 1/1000 dilution (500 bacilli) of the bovine strain. Five animals immunized with the B.C.G. culture were subsequently challenged and only one of them developed a lesion. Ten animals immunized with the H37Ra culture were challenged and two of them developed a lesion. In control groups, lesions have consistently developed after an interval of about 11 days. Both strains therefore produced some degree of immunity.

It is generally accepted that when B.C.G. is inoculated into animals the organisms multiply, whereas this does not occur with the H37Ra strain. It seemed of interest to determine the fate of these strains when inoculated into the cornea of mice. This was followed by both the phase contrast and gross staining methods.

Intracorneal inoculations of B.C.G. were made with undiluted cultures, as well as cultures diluted 1/10 and 1/100. These were followed by phase contrast examination as well as by gross staining. One day after inoculation, a light core of macrophages and leucocytes was seen. Many bacilli were found, all intracellular, in both types of cells. The bacilli were short and rather thick. By the 4th day no further cell invasion was apparent. The bacilli were now long and rather filamentous, but still within macrophages. On the 11th day the picture had changed. There was a dense core, mainly composed of " dots " and débris around which macrophages and leucocytes full of bacilli were seen ; there were also some extracellular bacilli. Apparently by this stage bacilli had overgrown and destroyed some cells. At the 7th week, phase examination showed several foci of cells with dots, but no bacilli were seen.

The multiplication of bacilli was followed by gross staining, by the method described by Robson and Sullivan (1957). There was no striking and rapid multiplication as seen with the bovine strain, but nevertheless, the data suggest that the bacilli had multiplied to about 10 times the original number by the 7th week after inoculation. It is incidentally of interest that large numbers of bacilli were present at that stage, even though they were no longer visible by phase contrast. Presumably they were masked by the cells and cell débris. Four corneas were examined at the 10th week and by that stage the bacilli retained the stain poorly, suggesting that degenerative changes had occurred. In summary there was a rapid but comparatively small cell invasion and slow multiplication of the bacilli.

The same methods were used to study eyes inoculated with an undiluted culture of H37Ra. Immediately after inoculation large numbers of bacilli were seen by phase contrast. One day later a few macrophages and leucocytes had invaded the needle track and only a few extracellular bacilli were found. At 3 days there were macrophages in the needle track but no bacilli were seen. At 6 days the cellular invasion was still small and macrophages containing fairly large numbers of bacilli were found. The appearance was similar in eyes examined at various periods up to 52 days after inoculation, though the impression was gained that the number of bacilli was decreasing. Gross staining showed that the number of bacilli appeared to remain constant for some time, and no evidence of any multiplication was found. Towards the end of the period of observation,

the number of bacilli seemed to be decreasing. No striking morphological changes, *e.g.*, beading, were seen in the bacilli. It is clear, therefore, that bacilli of this avirulent strain call forth little cellular reaction and do not multiply within the cornea of mice.

DISCUSSION

Three main criteria have been used experimentally to determine the protective value of immunization in tuberculosis, all involving accurate comparison between re-inoculated and control animals. They are :

1. Survival time ;
2. the condition of various organs (notably the lungs, liver and spleen) at a given time after the challenge infection ; and
3. the number of organisms that can be demonstrated at various times after infection.

These methods have yielded much information on the development and mechanisms of immunity, but they are laborious and suffer from the disadvantage that it is usually impossible to differentiate accurately between the organisms and lesions of the immunizing and of the challenging infections. Moreover, with virulent bacilli, the size of the immunizing dose is strictly limited, otherwise widespread lesions and death of the animal will preclude any accurate judgment of the degree of immunity.

Selbie and O'Grady (1954) studied immunity by inoculating two separate sites in mice, *i.e.*, intramuscular injection into the thighs. This method, however, does not appear to be very suitable for accurate quantitative work. Dubos and Schaefer (1956) were able to differentiate between the primary and challenging infections by using eugonic and dysgonic strains of virulent bovine tubercle bacilli.

In the present investigation a simple method is described by which the effect of immunization can be assessed quantitatively. The measurement is all or none, *i.e.*, the appearance of a macroscopic lesion in the cornea. The immunizing effect of any particular organisms can be demonstrated on a small group of mice, without the occurrence of open tuberculosis and risk of infection to personnel. In agreement with previous work (Robson and Didcock, 1956) it has been shown with this method that immunity can be produced not only by a virulent strain, but also by the attenuated strain (B.C.G.), and by an avirulent strain (H37Ra) which shows no evidence of multiplication on inoculation into the mouse cornea.

When the immunizing inoculation is made into one cornea of the mouse, and the challenge inoculation into the other cornea, the two infections may be followed independently. The pathological reactions and the fate and multiplication of the organisms can be followed at both sites. The immunizing infection can, however, be given in another part of the body and the present work has shown that it is effective when it is made intradermally. Goulding (1957*a*, *b*) has shown that such immunity can also be produced by the intravenous injection of B.C.G. and H37Ra in mice and subsequent challenge in the cornea.

The corneal method has also been used in studying the mechanism of immunity. There are some clear differences between the changes which occur in the immune cornea and those seen in a primary infection, namely :

1. The organisms in immune corneas show no significant multiplication, remaining intracellular, while in the primary infection there is rapid multiplication which leads to their overgrowth in macrophages and the appearance of a macroscopic lesion.

2. The cellular reaction to the inoculation in the immune eye is similar to that found in non-immune eyes but is less extensive. Moreover, there is no evidence of hypersensitivity response to the challenge inoculum in the second eye. This is indeed in agreement with previous findings in the mouse.

It is therefore clear that under the conditions of the experiments described, the protective action of immunity does not depend on a hypersensitivity reaction or on accelerated and intensified cellular response to the challenging inoculum. On the contrary, fewer cells aggregate in the immune cornea but nevertheless they are able to keep in check the multiplication of the bacilli. It may well be that the immunity is merely due to the ability of these cells to prevent bacterial growth, although the present experiments do not exclude the possibility that humoral factors are also involved. The question of cellular and humoral factors in immunity to tuberculosis is discussed by Raffel (1955) who emphasizes that conclusions may have to be drawn, not from experiments *in vitro*, but from observations on living tissues. The method described here offers an opportunity for such studies and is being used for this purpose.

SUMMARY

A method is described for the detection of immunity to tuberculosis in mice. The immunizing dose is injected intracorneally or intradermally and the subsequent challenging dose intracorneally. In the presence of immunity, no macroscopic corneal lesion (which occurs with a primary infection) is produced by the challenge infection.

The changes in the cornea following a primary and a challenging inoculation have been compared by various methods. In the immune eye there is less cellular reaction and no evidence of bacterial multiplication.

Immunity was induced not only by a virulent bovine strain of *Myco. tuberculosis* but also by B.C.G. and by H37Ra. Examination of corneas inoculated with these strains showed that B.C.G. and H37Ra produced less cellular reaction than the virulent strain. B.C.G. showed some evidence of multiplication but there was no multiplication of H37Ra in the cornea. Hence, this avirulent strain was capable of inducing immunity even though it did not multiply at the site of inoculation.

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A STUDY OF THE ANTIGENICITY OF BASEMENT MEMBRANE AND RETICULIN

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THE immuno-histological technique of Coons (Coons and Kaplan 1950 ; Coons 1956), which uses antibody protein conjugated with fluorescein as a stain for the corresponding antigen, has made possible the accurate localization in tissue sections of antigens native to the tissue (Marshall, 1951 ; Hill and Cruickshank, 1953 ; Mellors, Siegal and Pressman, 1955).

Using this technique, Cruickshank and Hill (1953*a*, *b*) demonstrated that an antiserum against a crude homogenate of glomeruli reacted *in vitro* with reticulin and basement membrane in the kidney and other organs. These observations indicate that there is some overlap of antigenic components between renal glomeruli and other tissues. They do not establish whether this overlap is complete (*i.e.*, that glomeruli and other tissues share a single antigen or an identical set of antigens) or partial. To resolve this question, experiments have been undertaken to compare the reactivity of antibodies evolved by immunizing animals with extra-renal tissues, with the reactivity of an antiserum against a crude homogenate of glomeruli. Synovium was selected for the first experiment on these lines and the purpose of this paper is to report a comparison of the properties of conjugated anti-glomerulus and anti-synovium antibodies, to present evidence for the presence in the glomerulus of two distinct antigens (or groups of antigens), and to describe the distribution of these antigens in the body.

Globulin fractions prepared from the sera of rabbits immunized with isolated human renal glomeruli or with human synovial membrane were conjugated with fluorescein by the method of Coons and Kaplan (1950). Unfixed sections of healthy human organs and tissues were examined under the fluorescence microscope after exposure to one or other of the conjugated antisera. In each case certain tests of specificity (*vide infra*) were carried out. The distribution of the specific fluorescence produced by one conjugate was compared with that produced by the other. Cross-inhibition experiments in which sections were pre-treated with the unconjugated anti-glomerulus globulin before exposure to the conjugated anti-synovium globulin (and vice versa) were performed to compare the antibody content of the two antisera.

MATERIALS AND METHODS

Preparation of homogenate and glomeruli

Glomeruli were isolated by a method based on that of Krakower and Greenspon (1951) from histologically normal human kidneys obtained at autopsy and stored at − 15° until required. The isolated glomeruli were suspended in buffered saline (pH 7·2) containing 1/10,000 merthiolate. The suspension, containing naked glomerular tufts, empty parietal capsules, whole glomeruli and clumps of tubule cells, was fragmented in a Griffith tube,

packed by centrifugation at 3000 r.p.m. for 30 min., and finally adjusted to a 10 per cent v/v concentration in buffered saline. Tubule cells formed up to 10 per cent of the suspension before fragmentation. No attempt was made to estimate the proportions of whole glomeruli and glomerular derivatives.

Preparation of homogenate of synovium

Human synovial membrane was obtained at operations for internal derangement of the knee joint and at autopsy from the knee joints of cases of accidental death.

The synovial membrane was freed as far as possible from sub-synovial tissue by blunt dissection and the material remaining, which consisted of synovial cells, capillaries and sub-synovial tissue, was pooled and stored at − 15° until required.

The antigen suspension was prepared by grinding pooled synovium in sand. The sand was separated by sedimentation in buffered saline. The ground synovium was packed in the centrifuge, re-suspended in buffered saline, fragmented in a Griffith tube, packed by centrifugation and finally brought to a 20 per cent v/v suspension in buffered saline.

The antigen was injected into rabbits as a 10 per cent v/v suspension in saline and also (following the method of Freund) as a 10 per cent water in oil emulsion, containing killed tubercle bacilli, made up as follows : 5 ml. of 20 per cent suspension of synovium in saline were emulsified with 5 ml. of liquid paraffin in which 25 mg. of killed tubercle bacilli had previously been incorporated. Crill 19 (Croda Ltd., Snaith, Yorks) was used as an emulsifying agent.

Preparation of antisera

Suspensions of antigen in saline were injected intra-peritoneally in 1 ml. doses on 4 days of the week for 4 weeks.

The antigen-adjuvant emulsion in an immunizing dose of 2 ml. was injected subcutaneously in four separate sites, one in each limb on a single occasion (Table I).

TABLE I.—*Immunization Schedules*

Rabbit	Antigen in saline 4-week courses	Antigen + adjuvants 4 × 0·5 ml. injections	Antigen
H/124	2	—	Glomeruli
H/125	2	—	,,
H/131	2	1	Synovium

Conjugation of globulins with fluorescein

The animals were bled from an ear vein 10 days after completion of immunization. Conjugates were prepared, by the method of Coons and Kaplan (1950) from serum globulin separated by half saturation of serum with ammonium sulphate. Before use conjugates were absorbed twice with an equal volume of packed washed human Group O red cells, twice with normal human serum and twice with acetone-dried guinea-pig liver powder (to remove the non-specific staining produced by all untreated conjugates). The absorption with human serum was carried out by adding one part of normal human serum to 100 parts of the conjugate. The serum and conjugate were allowed to react for 12–24 hr. at 4° ; the mixture was then centrifuged and the supernate kept for use.

Preparation of tissue sections

The methods of Coons and Kaplan (1950) and Coons, Leduc and Kaplan (1951) were followed in the preparation of quick-frozen unfixed blocks of tissue and in the cutting of sections (4 μ thick) from them in a cryostat. The sections were mounted on acid-cleaned glass slides without adhesive. After staining in one or other of the ways described below, the sections were mounted in glycerol containing 1 part in 10 of buffered saline, and covered with a glass coverslip.

Fluorescence microscope

The light source and filters used were as described by Hill and Cruickshank (1953), except that the gelatine and sodium nitrite eye-piece filters were replaced by a Lempert filter.

When a photograph of a stained section was to be taken, slides made from OX1 filterswere used in place of ordinary glass slides. Their use increases contrast by replacing glass slides, which produce a blue background fluorescence, with non-fluorescent material. They also absorb some of the blue-violet light transmitted by the Woods glass filter, positioned between the light source and the microscope. Coverslips made of OY12 glass were used in conjunction with the OX1 filters to prevent fluorescence of the microscope lens system excited by ultra-violet light entering the objective (the use of OX1 filters and coverslips of OY12 glass was suggested by Dr. W. R. Alexander).

Staining experiments

The staining of tissue sections by fluorescein-conjugated antibody globulin is in essence an immunological reaction resulting in the fixation of an antibody by its antigen in the tissue. The site of fixation of a fluorescein-conjugated antibody is rendered readily visible by virtue of its yellow-green fluorescence when exposed to ultra-violet light.

Direct staining experiments

In this group of experiments staining produced by the anti-glomerulus conjugate was compared with that produced by the anti-synovium conjugate. Two contiguous sections from the same block of tissue were mounted on one slide. Each section was pre-treated for 2 hr. with unconjugated normal rabbit globulin, rinsed in buffered saline and exposed to one or other of the conjugates for 1½ hr., after excess saline had been carefully removed from the slide. Exposure to the conjugates was followed by a wash for a total time of 10 min. in two changes of buffered saline which was kept gently agitated by a stream of air bubbles.

Tests of specificity

The criterion of specificity of staining was the demonstration that unconjugated antibody globulin would block the fluorescence produced in a tissue section by the corresponding conjugate, whereas unconjugated normal rabbit globulin would not. Of two sections mounted on the same slide, one was pre-treated with normal rabbit globulin (2 hr.), and the other with unconjugated antibody globulin (2 hr.), and both were subsequently exposed to the conjugate under investigation for 1½ hr. Such tests of specifcity were routinely applied.

Cross inhibition experiments

These were performed in the same way as the tests of specificity except that sections were pre-treated with one antibody globulin (unconjugated) and then exposed to a conjugated fraction of the other antibody globulin. Direct staining with each of the conjugates and tests of specificity were run in parallel with cross inhibition experiments.

RESULTS

Direct staining experiments

The observations made in this group of experiments are set out in Table II. No attempt has been made to record variations in intensity of fluorescence produced in sections stained by different conjugates, or between individual elements in the same preparation. The factors governing the intensity of fluorescence produced are not readily controlled so that a reliable estimate of the relative strengths of two antibody solutions or the relative concentrations of two antigenic components in the tissues cannot be made from a visual comparison of the intensity of fluorescence produced.

Only the anti-glomerulus conjugate produced staining of the renal glomerular capillaries (Fig. 2), the basement membranes of the thyroid acini (Fig. 8), and the media of arteries (Fig. 5). On the other hand both conjugates stained the basement membrane of renal tubules (Fig. 2), sarcolemma (Fig. 4), and the periacinar reticulin in the thyroid gland (Fig. 8 and 9). Both stained fine fibrils lying in the interstitium of the thyroid gland and among the collagen fibres of the splenic

TABLE II.—*Results of Direct Staining and Cross Inhibition Experiments*

Organ	Histological component	Direct staining experiments: Anti-glomerulus conjugate	Direct staining experiments: Anti-synovium conjugate	Cross inhibition experiments: Anti-glomerulus globulin and anti-synovium conjugate	Cross inhibition experiments: Anti-synovium globulin and anti-glomerulus conjugate
Kidney	Glomerular capillaries	+	−	−	+
	Glomerular epithelial cells	+	+	−	−
	Parietal capsule of glomerulus	+	+	−	−
	Tubular basement membrane	+	+	−	−
	Tubular cells	−	−	−	−
	Peritubular capillaries	+	+	−	−
	Media of arteries	+	−	−	+
Thyroid	Acinar basement membrane	+	−	−	+
	Peri-acinar reticulin	+	+	−	−
	Interstitium	+	+	−	−
	Capillaries	+	+	−	−
	Media of arteries	+	−	−	+
Spleen	Reticulin	+	+	−	−
	Fibrils in trabeculae	+	+	−	−
	Media of arteries	+	−	−	+
	Sarcolemma	+	+	−	−

trabeculae. Both stained sub-synovial capillaries and both produced specific pericellular fluorescence in the synovial cell layer (Fig. 6 and 7). Neither conjugate stained collagen.

The anti-synovium conjugate did not stain any structures which were not also stained by the anti-glomerulus conjugate.

The most striking difference between the two conjugates was seen in their reaction with the renal glomerular tufts (Fig. 1). The anti-glomerulus conjugate, which stained the basement membrane of glomerular capillaries, produced a characteristic picture made up of a series of fluorescent loops. The anti-synovium conjugate did not produce a loop pattern in glomeruli but it stained numerous discrete structures, round, oval, polygonal, or crescentic in shape, in the glomerular tuft. Under high power these structures frequently contained a round unstained centre. The appearances suggested that it was the cytoplasm of cells which had reacted with the conjugated anti-synovium preparation. Occasionally two or three such cells lay side by side forming the boundary of an unstained space. Similar cells could be seen in preparations stained with the anti-glomerulus conjugate. In this case they lay closely applied to the basement membrane of glomerular capillaries. No firm conclusion about the relationship of these cells to basement membranes could be formed. It follows that the identity of these cells has not been firmly established but their situation suggests that they may be glomerular epithelial cells. Very occasionally similar cells were seen lying in Bowman's capsule.

Cross inhibition experiments

Cross-inhibition experiments were performed to confirm the differing antibody content of the two types of antisera. The results are summarized in Table II. It was found that unconjugated anti-glomerulus globulin would inhibit all the staining produced by anti-synovium conjugate, whereas unconjugated anti-synovium globulin would prevent only some of the staining produced by anti-glomerulus conjugates. The anti-synovium globulin failed to prevent the reaction of anti-glomerulus conjugate with the basement membranes of glomerular capillaries (Fig. 3) and with the media of muscular arteries and arterioles. It did prevent the reaction of the anti-glomerulus conjugate with extra-glomerular capillaries, renal tubular basement membrane and the sarcolemma of muscle. A section of thyroid gland pre-treated with the anti-synovium globulin (unconjugated) and subsequently exposed to the anti-glomerulus conjugate, showed fluorescence lying only in the position of a basement membrane (Fig. 10: compare with Fig. 8 and 9).

EXPLANATION OF PLATES

All the photographs are fluorescence photomicrographs of unfixed tissue sections treated with fluorescent globulin solutions. The lighter lines and areas in the photographs represent the yellow-green fluorescence of fluorescein and indicate sites of fixation of conjugated antibody. Most tissue components exhibit a dull blue-grey autofluorescence under the fluorescence microscope. Such autofluorescence is not readily confused in the photographs with the fluorescence of fluorescein. Elastic tissue exhibits a brilliant white autofluorescence which has the same appearance in the photographs as green specific fluorescence.

FIG. 1.—Upper. Human renal glomerulus treated with anti-glomerulus globulin. Specific fluorescence in basement membranes and epithelial cells. (× 350.)

Lower. Human renal glomerulus treated with anti-synovium conjugate. Staining confined to the epithelial cells. The cell seen in the lower right-hand corner lies on Bowman's capsule. (× 350.)

FIG. 2.—Human kidney treated with anti-glomerulus conjugate (top), and with anti-synovium conjugate (bottom). Specific fluorescence occurs in tubular basement membrane in both cases. (× 120.)

FIG. 3.—Human kidney. The section was pre-treated with unconjugated anti-synovium globulin and stained with anti-glomerulus conjugate. Specific fluorescence is present only in the glomerular tuft. (× 100.)

FIG. 4.—Human muscle treated with anti-synovium conjugate. Specific fluorescence is present in the adventitia of the vessel and in the sarcolemma. The white spots in the media of the artery and in the muscle represent granules of pigment showing orange autofluorescence. (× 350.)

FIG. 5.—Splenic artery treated with anti-glomerulus conjugate (G) and with anti-synovium conjugate (S). The arrow lies at the junction of the composite photomicrograph and points to the internal elastic lamina, which has a brilliant white autofluorescence. Only the antiglomerulus conjugate has reacted with the media of the vessels. Specific fluorescence occurs in the adventitia and splenic reticulin in both cases. (× 150.)

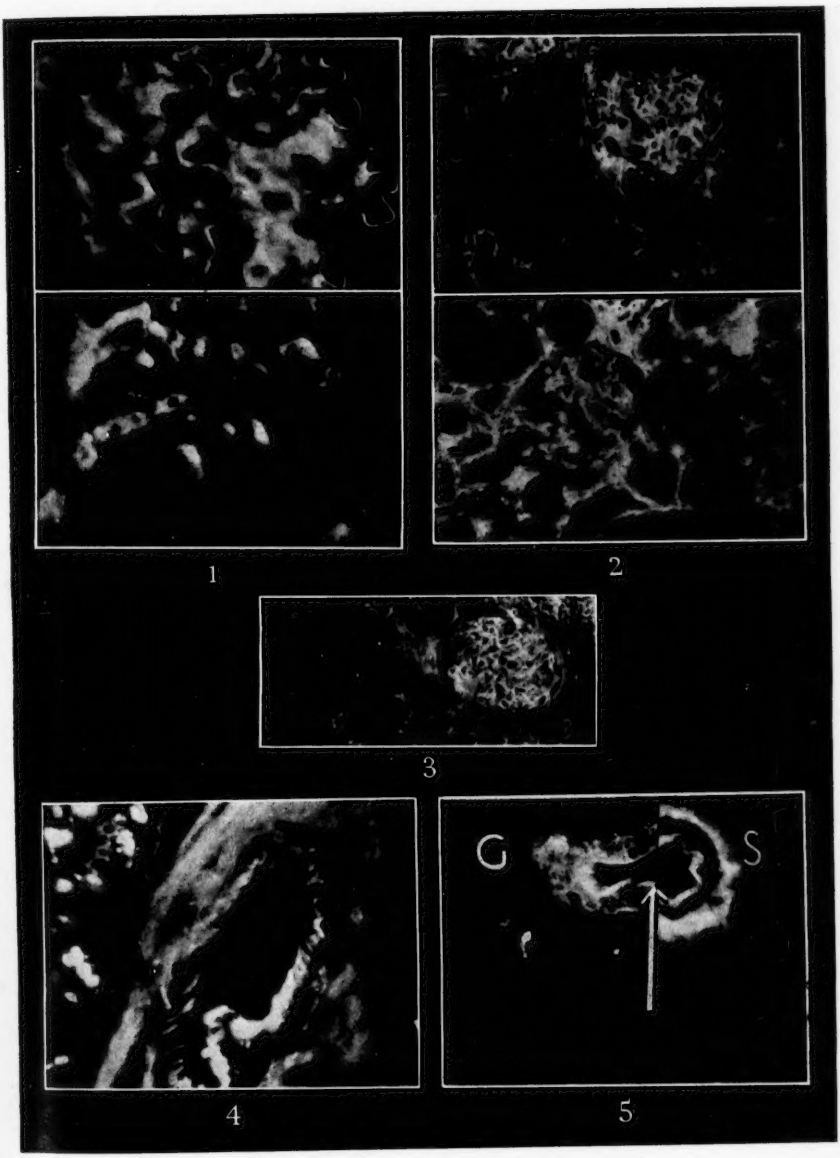
FIG. 6.—Synovium treated with anti-glomerulus conjugate. Specific fluorescence is present around the synovial cells and in the sub-synovial capillaries. (× 250.)

FIG. 7.—Synovium treated with anti-synovium conjugate. The specific fluorescence has the same distribution as in Fig. 6. (× 250.)

FIG. 8.—Thyroid treated by anti-glomerulus conjugate. The green fluorescence of the basement membrane is represented by a thick white line. The periacinar reticulin lying beneath the basement membrane is also specifically stained. (× 200.)

FIG. 9.—Thyroid treated with anti-synovium conjugate. The periacinar reticulin appears as a single line which splits to enclose round unstained spaces. (× 250.)

FIG. 10.—Thyroid pre-treated with unconjugated anti-synovium and stained with conjugated anti-glomerulus. Only the basement membrane is stained. (× 200.)



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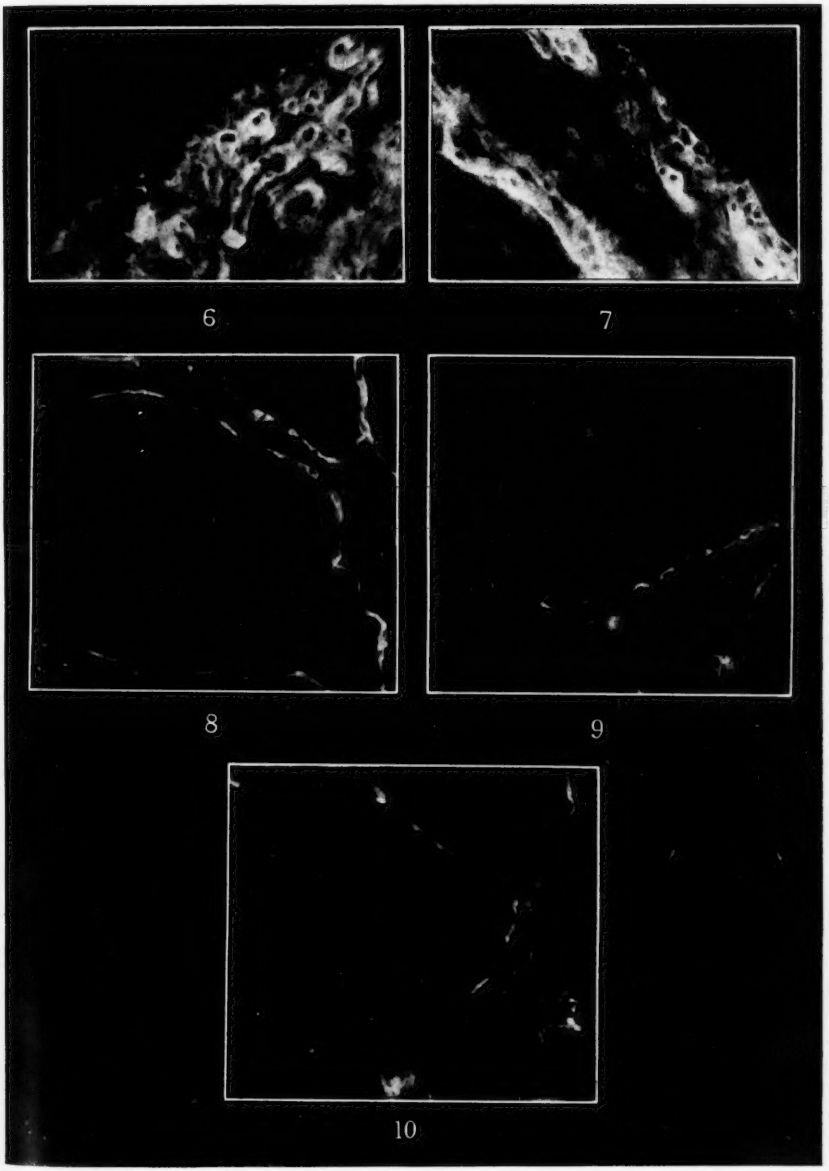

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DISCUSSION

The reactivity of the antiserum to isolated human glomeruli was the same as that described by Cruickshank and Hill (1953*a*) in that it was fixed *in vitro* by reticulin and basement membrane in the kidney and other organs. Mellors *et al.* (1955), using an *in vitro* technique, found a more limited uptake of anti-kidney antibody. They injected rabbit antisera to rat kidney into rats during life and traced the distribution of the injected antibody after death by means of conjugated chicken anti-rabbit globulin. These observations, based as they were on experiments *in vivo*, cannot be directly compared with those of Hill and Cruickshank. The differences may reflect variations in accessibility of the antigen to the antibody, which in one case is brought to the intact kidney by the blood stream, and in the other is applied to tissue sections.

The present observations suggest that the renal glomerulus contains at least two antigens. One of these, present in the basement membrane of the glomerular capillaries, has been shown to be shared by basement membrane in other organs. A study of the distribution of this antigen throughout the body has not yet been completed but preliminary observations indicate that it is present in the choroid plexus, the intra-hepatic bile ducts, the thyroid gland, and in the media of muscular arteries and arterioles, but not in the media of elastic arteries. The other glomerular antigen is more widely distributed throughout the body, and is found in much the same sites as argyrophilic reticulin.

It should be borne in mind that the preparations used for immunization in these experiments were crude tissue homogenates. Although the resulting antisera enabled certain tissue elements to be distinguished from each other, it is not justifiable to assume that each group so distinguished contained a single identical antigen. Such an assumption could only be made if the antisera were prepared by immunization with a single chemically pure antigen. For simplicity of description the term antigen is used here in circumstances where " antigen or group of antigens " would be more accurate.

Physical separation of tissue components differing in antigenic specificity has been accomplished by Goodman, Greenspon and Krakower (1955). They used a flocculation technique to study the antigenic inter-relationships of the various anatomical components of the dog's kidney isolated by sonic vibration and they identified two groups of antigens in the glomerulus. One of these was capable of stimulating the production of a heterologous nephrotoxic anti-serum. The other group of antigens, which did not evoke a nephrotoxic antiserum, was apparently present also in the glomerular parietal capsules and in a fraction consisting of the basement membranes of tubules (and stroma from the cortex) as well as in collagenous fibrils derived from tendon and cornea. The present observations are largely in agreement with those of Goodman *et al.* in that all these tissues specifically fixed an antibody against synovium which reacted also with certain elements (not including the capillary basement membrane) in the glomerulus.

More recently Baxter and Goodman (1956) have shown that an antigen capable of evoking a heterologous nephrotoxic serum is present in the renal medulla, lung and placenta of the rat in about the same concentration as in the renal glomerulus, while other organs (intestine, heart, liver stroma) contain this antigen in smaller amounts.

The present observations indicate that an organ rich in one antigenically active component of connective tissue is not necessarily rich in the other. It is not possible to relate the concentration of the nephrotoxic antigen, as defined by Baxter and Goodman, in individual organs of the rat, with the distribution in homologous human organs of either of the glomerular antigens demonstrated by the present application of Coons' technique. If the present observations could be taken to show that the glomerulus contains only two antigenically active components, this comparison might be possible. However, they only indicate that there are at least two such components. The antigenic relationship between capillaries and reticulin suggested by the present observations has not been finally established. Both capillaries and reticulin reacted with an antibody to a homogenate of synovium, but the latter contained capillaries which may have evoked an antibody distinct from that which reacted with reticulin.

The results of the present investigation and those of Goodman and his co-workers indicate that connective tissue is an antigenically heterogeneous substance. A study of the antigenicity of the various components of connective tissue and of the distribution throughout the body of the antigenically related components may help towards a better understanding of the pathogenesis of the diffuse mesenchymal diseases. The technique of Coons, which enables antigenically active components of connective tissue to be accurately localized, should prove a useful supplement to chemical and enzymic studies of connective tissue.

SUMMARY

The immuno-histological method of Coons was used to study the distribution of the antigenically active components of human synovial membrane and of human glomeruli.

Reticulin and basement membrane appear to be antigenically distinct. While the renal glomerulus was shown to possess both antigens, only the reticulin antigen was demonstrated in the synovial membrane and only the basement membrane antigen in the media of arterioles.

The relationship between these findings and those of other workers is discussed. It is suggested that the immuno-histological technique of Coons should prove to be a useful supplement to chemical and enzymic studies on connective tissue, and that a study of the distribution throughout the body of the several antigenically active components of connective tissue might help towards an understanding of the sites of the lesions in the diffuse mesenchymal diseases.

I am most grateful to Dr. A. G. S. Hill for patient tuition in the use of Coons' technique and to Dr. Hill and Dr. C. L. Greenbury for their helpful comments during the preparation of the script for publication.

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INDUCTION BY AMINOACETONITRILE OF A PREDISPOSITION TO GIANT CALLUS FORMATION

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In certain individuals bone fractures tend to result in the formation of gigantic calluses. The so-called " callus luxurians " is well known in clinical pathology and, although its aetiology is unknown, it has been vaguely ascribed to some constitutional predisposition. The careful survey of the literature by Lauche (1937) has revealed, furthermore, that occasionally tumours (osteomas, chondromas or even sarcomas) may develop at fracture sites in patients who are predisposed to excessive callus formation.

Previous studies concerning the skeletal changes induced in rats by treatment with aminoacetonitrile (AAN) suggested to us that this drug may predispose to giant callus formation. AAN is an analogue of the β(γ-L-glutamylamino) propionitrile which occurs in the seeds of *Lathyrus odoratus* and presumably accounts for the toxic effects of this plant on the skeleton (Dupuy and Lee, 1954, 1956 ; Schilling and Strong, 1954). Both these nitriles, when fed or injected into rats, produce numerous exostoses, especially at muscle insertion sites, and an irregular proliferation of cartilage, particularly in the rapidly growing epiphyseal plates. Although the presence of AAN has not been demonstrated in *Lathyrus odoratus* seeds, it is even more effective than the naturally occurring β(γ-L-glutamylamino)propionitrile (Wawzonek, Ponseti, Shepard and Wiedenmann, 1955). The literature on bone lathyrism has been the subject of an excellent review (Strong, 1956), so that we need not discuss it here in detail. Suffice it merely to add that more recently we have learned that the response of bone, either to diets containing *Lathyrus odoratus* seeds, or to subcutaneously administered AAN, can be influenced at will. For example, cortisol (hydrocortisone), ACTH and thyroxine inhibit, while STH or partial hepatectomy augment, the characteristic bone lesions produced by lathyrogenic substances (Selye, 1957*a* ; Selye and Bois, 1956*a*, *b*, 1957*a*, *b*). In the course of these investigations, we noticed that rats affected by severe bone lathyrism often suffer spontaneous fractures in which callus formation appears to be excessive. This tendency to fracture and giant callus formation was influenced, in the same sense as the bone lathyrism itself, by the hormones just mentioned and by hepatectomy. However, no definite conclusions could be drawn from these incidental observations, because our untreated controls, which received no AAN—as well as the rats which, despite AAN treatment, developed no skeletal changes, because of the concurrent administration of cortisol, ACTH or thyroxine—did not suffer spontaneous fractures which might have served for comparison. It was therefore decided to undertake a systematic comparative study of the response of bone to a standard trauma in untreated and in all AAN-treated rats.

EXPERIMENTAL

Forty female Sprague-Dawley rats, having an initial body weight of 112 g. (range : 100–118 g.), were subdivided into two equal groups. Group I acted as controls, while Group II was treated with 10 mg. of AAN, administered in the form of aminoacetonitrile hydrosulphate, in an aqueous solution containing 25 mg./ml. This daily dose was injected subcutaneously in two portions of 5 mg. each, at 9.00 a.m. and at 5.00 p.m., over a period of 12 days. Injections were then discontinued, in order to permit partial consolidation of the fracture sites. Preliminary experiments had shown that it is difficult to dissect a callus from the surrounding soft tissues while AAN administration is still in progress, because the bones are too soft and fragile. However, in order to follow the chronological development of the callus, two rats of each group were killed on the 8th, 10th and 12th day, respectively, and the remaining animals were sacrificed 5 days after the last AAN injection.

Bone fractures were made in both groups on the first day of the experiment, as follows : the skin was shaved in the inguinal region and then, under ether anaesthesia, an incision about 2 cm. in length was made through the skin, just above the inguinal canal. The skin was then pulled down to the level of the upper end of the tibia ; this can easily be done in the rat because of its loose subcutaneous connective tissue. (An indirect approach was used in order to minimize the danger of infection, which is always great when a bone fracture is placed just under a skin incision.) The proximal third of the tibia was then freed of the surrounding muscles and transected with scissors 3 mm. above the insertion of the fibula. In the rat, the distal end of the fibula is rigidly connected with the tibia by a synostosis ; consequently, the fibula acted as a splint which maintained the two ends of the tibial fracture in position. After severance of the bone, the skin was closed with two sutures.

In order to compare the healing of wounds in bone and in cartilage, immediately after completion of the operation on the tibia, we also made a 2-mm. incision, longitudinally through the purely cartilaginous caudal end of the xiphoid. This was exposed, while the animal was still anaesthetized, through a transverse incision 1 cm. in length, across the shaved skin in the upper epigastric region.

Immediately after autopsy, the tibia and fibula of each rat were fixed, and simultaneously decalcified, in Susa solution, for subsequent histological examination. After being embedded in paraffin, sections 6 μ in thickness, were stained either with haematoxylin-eosin or with a modification of Gomori's (1950) aldehyde-fuchsin technique.* In the xiphoid cartilage, the wound healed without callus formation and hence this tissue was not preserved for histological study.

RESULTS

As early as the third day after the trauma, even mere digital palpation showed that the callus became much more voluminous in the AAN-treated than in the control rats. Dissection of the bones of the animals killed at intervals during the experiment merely confirmed this observation. The extraordinary proliferation of bone around the fracture sites was particularly striking in rats killed at the end of the experiment, following a 5 days' rest from AAN treatment (Fig. 1). By that time, in the controls, the callus was rigid but small, while in all of the AAN-treated rats, an enormous amount of bone tissue had developed, especially below the level of the fracture, and even the interosseous ligament was largely ossified. On the other hand, despite all this excessive bone formation, the union between the cut bone ends was delayed, because the wound surfaces themselves remained connected only by soft tissues, in which ossification was retarded.

Histological examination also showed a definite difference between the structure of the calluses in the two groups. The proliferation of fibrous, osseous and cartilaginous tissue was much less pronounced in the controls than in

* We found that the bone corpuscles characteristic of experimental lathyrism can best be demonstraed by first staining paraffin sections with haematoxylin and then, mordanting them with 1 per cent oxalic or 0·8 per cent periodic acid for 5 min. prior to colouring with the aldehyde-fuchsin technique of Gomori.

the AAN-treated animals. In the latter, the union between the traumatized bone surfaces was retarded by the excessive development of fibro-cartilaginous tissue, which led to a gradual displacement of the upper bone stump, to behind and below the proximal extremity of the lower (Fig. 2). While, in the wound itself, ossification was thus delayed, there developed a great excess of cancellous bone along the entire circumference of the tibia both above and below the fracture. On the specimens obtained after 8 and 10 days of AAN treatment this cancellous bone was only partially calcified, but was otherwise essentially normal in appearance ; however, in the rats killed at the end of the experiment, the characteristic basophilic and aldehyde-fuchsin-positive corpuscles of experimental lathyrism —which have been described in a previous communication (Selye, 1957*b*)—were already quite obvious (Fig. 3 and 4). These varied in size from dust-like just visible particles up to structures having a diameter of about 1 μ, and they occurred most frequently at places where cartilage was undergoing direct metaplasia into bone. The spaces between the cancellous bone spicules were filled with partly fibrous, partly myeloid bone-marrow.

Interestingly, no excessive callus formation was noted in the traumatized xiphoid cartilage. Although cartilaginous elements grew excessively in the region of the tibial fracture, trauma to the purely cartilaginous portion of the xiphoid led to no excessive repair phenomena, and healing was virtually complete at the time of autopsy in both groups.

DISCUSSION

It is evident that AAN induces some metabolic disturbance, which predisposes the rat to the formation of luxuriant giant calluses, following experimental bone fracture. In and around these calluses, fibrous tissue, cartilage and bone proliferate in excessive amounts. Yet, the union of the fractures is delayed, because the newly formed tissue possesses a comparatively soft consistency and true ossification is inhibited particularly in the wound itself.

The excessive growths of bone resemble osteomas, but it would be difficult to decide whether they are actually benign tumours of the bone, or merely abnormal hyperplasias.

The mechanism of this overabundant callus formation is not clear. However, AAN appears to produce some systemic metabolic change, which predisposes

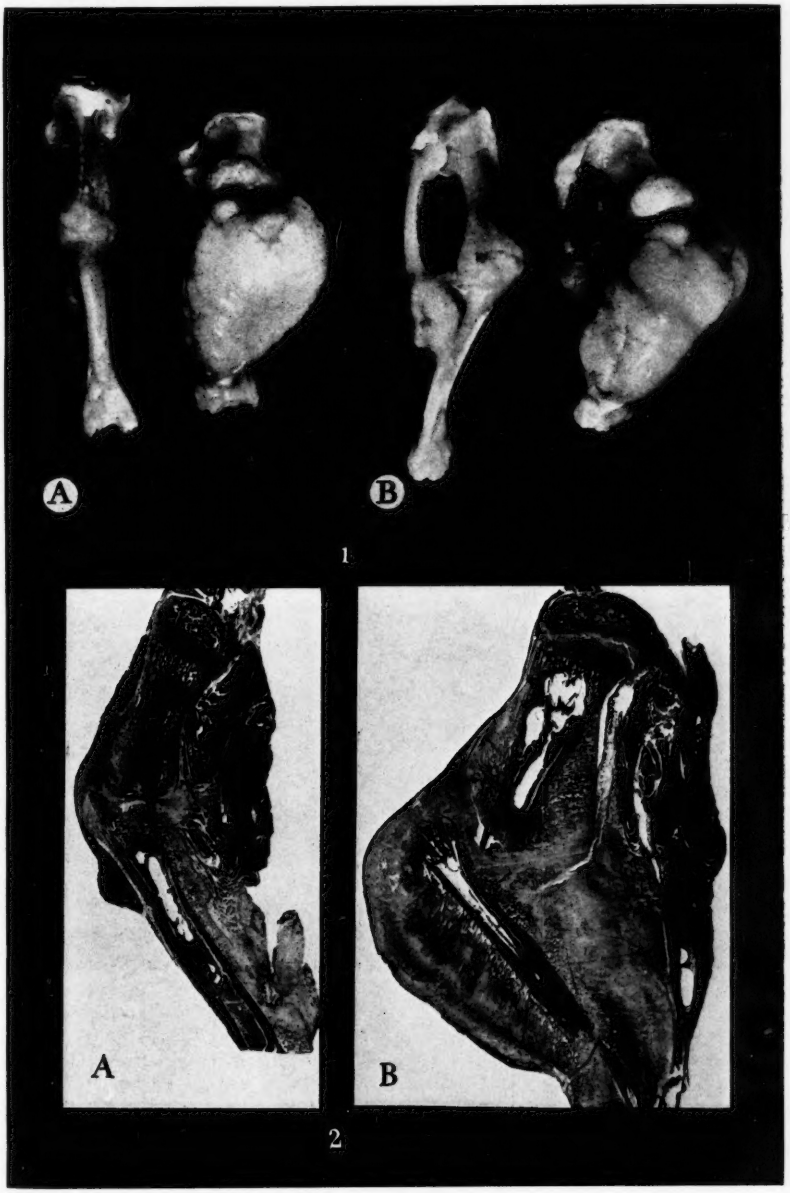
DESCRIPTION OF PLATES

FIG. 1.—Frontal (A) and lateral (B) view of the calluses on the tibias of one typical control (left) and one AAN-treated (right) rat. In the control, the fracture is solidly united by a small callus. In the AAN-treated animal, there is much soft connective tissue (dark area) between the stumps, while the callus assumes the appearance of an enormous multilobar osteoma and the interosseous ligament is likewise largely ossified.

FIG. 2.—Low magnification of a longitudinal section through the fractured tibia of a control (A) and an AAN-treated rat (B). In the control, the bone stumps are united, partly by fully ossified tissue. In the AAN-treated animal, connective tissue separates the bone stumps, the lower one of which has slipped forward. The excessive formation of light radially arranged periosteal bone spicules is clearly visible (aldehyde-fuchsin × 2).

FIG. 3.—Newly formed bone spicule of a control rat (top) in comparison with the corresponding region in the bone of an AAN-treated animal. Darkly staining intercellular globules are plentiful only in the latter animal, though occasional faintly staining structures of a similar type are also visible in the control (aldehyde-fuchsin × 240).

FIG. 4.—Cartilaginous tissue of periosteal origin in a healing bone fracture of a control (top) and an AAN-treated animal. Here again the darkly staining globules are clearly visible only in the AAN-treated rat (aldehyde-fuchsin × 240).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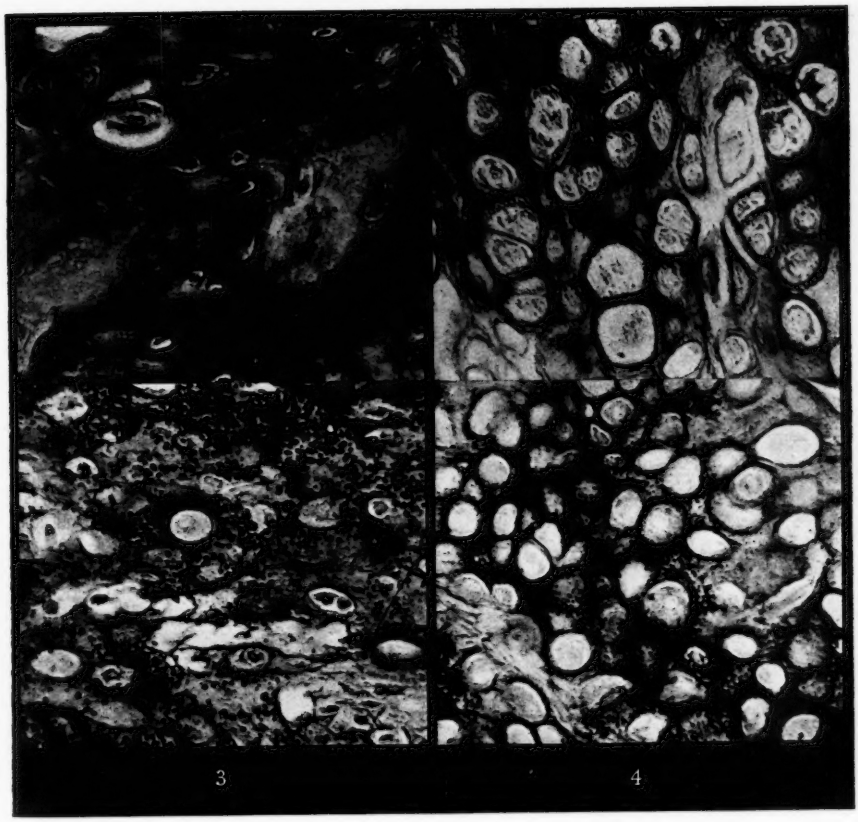

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bone to excessive reactions to injury, similar to the way in which the connective tissue of the skin is sensitized in people predisposed to keloid formation. Under the influence of AAN, excessive bone appears to develop wherever there is some special mechanical stress, for example, at muscle insertion sites. Presumably, the experimental bone fractures also stimulate osteogenesis through the production of topical stress. In this connection, it is interesting that the insertion of the fibula on the tibia—which becomes a point of special stress after transection of the latter bone—underwent hyperplasia even in our control animals, but osteogenesis at this point was incomparably greater in the AAN-treated animals (Fig. 1). Here again, the combination of local stress and sensitization by AAN is presumably of pathogenic importance. The presence of a great number of basophilic and aldehyde-fuchsin-positive bone corpuscles is the only anomaly, in the intercellular substance of bone and cartilage, characteristic of AAN intoxication. But it remains to be seen whether these bodies play a rôle in the production, or are merely the consequence, of lathyritic bone lesions.

It is hoped that sensitization by AAN for the production of giant calluses may furnish a technique suitable for the experimental analysis of the constitutional factors which play a part in the production of the " callus luxurians", the non-union fracture and the development of osteomas (especially those which tend to occur at muscle insertion sites in certain types of myositis).

SUMMARY

Experiments on rats indicate that systemic treatment with aminoacetonitrile (AAN) produces a metabolic defect, which causes the skeleton to respond with excessive callus formation following local injury. The healing of wounds situated in purely cartilaginous structures (such as the distal end of the rat's xiphoid) is not affected thus by AAN.

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A NEW CULTURE METHOD DESIGNED FOR KINETIC STUDIES ON DIPHTHERIA TOXIN PRODUCTION*

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It has been shown that several variants of the Park Williams No. 8 strain of *Corynebacterium diphtheriae* will grow and produce high yields of toxin in chemically defined media consisting of maltose, certain amino acids, vitamins and salts (Pappenheimer, Mueller and Cohen, 1937 ; Drew and Mueller 1951). Recently we isolated a variant of the P.W. No. 8 strain (SM1), exacting for only two amino acids, L-methionine and L-cystine (Yoneda, 1950). Using the SM1 strain, good yields of toxin were produced in the minimal medium (Yoneda, 1951*a*, *b*).

In the studies referred to above, the bacteria were grown as a pellicle on the surface of the medium. More recently it has become possible to produce diphtheria toxin in submerged or shaken culture (Linggood and Fenton 1947 ; Mitsuhashi, Kurokawa and Kojima, 1949). Using complex media, the method has proved useful for rapid large-scale toxin production (Linggood, Matthews, Pinfield, Pope and Sharland, 1955). No attempt seems to have been made to produce toxin in a chemically defined medium in submerged or shaken culture.

In the present paper a method is described for production of toxin in high yield in shaken culture using the SM1 strain and a medium containing only three amino acids. The method has proved useful for the rapid preparation of highly purified toxin, in following the kinetics of toxin production (Yoneda and Pappenheimer, 1957), in studies on amino acid metabolism of the organism and as reported in the following paper (Pappenheimer and Yoneda, 1957), for the rapid preparation of highly purified internally labelled ^{35}S-methionine and ^{35}S-cystine toxin.

MATERIALS AND METHODS

*SM*1 *Strain of* C. diphtheriae.

SM1 was isolated by repeated subculture of the Toronto variant of the P.W. No. 8 strain in the minimal medium (AMC) of Yoneda (1950). SM1 is exacting for L-methionine and L-cystine, although the latter can be replaced by sodium thioglycollate. A third amino acid, either asparagine or glutamic acid, must be used as a major nitrogen source. The culture has been maintained by subculture on AMC medium at 32–33°, without loss of toxigenicity, since 1951. It can also be stored in the lyophilized state.

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Preparation of Culture Medium

The composition per litre of AMC medium, as recently modified, is as follows. L-asparagine 5·0 g., L-methionine 0·2 g., L-cystine, recrystallized (20 per cent solution in 2 N-HCl) 2·0 ml., KH_2PO_4 5·0 g., NaCl 4·0 g., $CaCl_2.2H_2O$ (50 per cent solution) 2·0 ml., sodium DL-lactate (60 per cent Merck reagent) 3·0 ml., sodium succinate $6H_2O$ 3·0 g., de-ferrated maltose (50 per cent solution in 0·5 per cent $Na_2HPO_4.12H_2O$), 40 or 80 ml., vitamins and salts, solution II (Mueller and Miller, 1940) 2·0 ml. (The medium used in Step 3 (see below) was fortified with 500 μg. calcium pantothenate/l. medium.

The asparagine and methionine, salts, succinate and lactate are dissolved in distilled water and made up to 1 l. For removal of iron, the pH is adjusted to 7·4 with 20 per cent NaOH solution and 1·5 ml. of $CaCl_2$ solution is added. The medium is boiled 2–3 min. and filtered while hot : 0·4 ml. of $CaCl_2$ solution is added to the cooled filtrate, and the medium is again boiled gently for 5 min. with frequent shaking. The filtrate is cooled and tested for iron with *o*-phenanthrolene. It should give no detectable colour with this reagent, and the pH should be 7·0–7·1. Cystine and solution II are then added, the medium dispensed into flasks and autoclaved at 115° for 10 min. Before inoculation, the required amounts of 0·01 per cent $FeSO_4.7H_2O$ solution in 0·1 per cent HCl (autoclaved at 115° for 5 min.) and of de-ferrated maltose solution are added. The 50 per cent maltose solution is de-ferrated by adding 1 ml. of 50 per cent $CaCl_2$ solution per 100 ml. and boiling gently for 2–3 min. After filtration the solution is sterilized at 115° for 5 min.

Culture Method.

The method consists of three steps as follows : 1. Cultivation of the inoculum in AMC medium of low iron content up to the beginning of the declining growth phase. 2. Harvest of inoculum by centrifugation and washing with iron-free medium. 3. Cultivation of washed concentrated cells in iron-free medium.

Step 1.—A large loopful of pellicle of SM1 strain is transferred to a small volume of AMC medium (20–25 ml. containing 2 per cent maltose and 10–25 μg. $FeSO_4.7H_2O$ per 100 ml.). After 15–16 hr. growth at 32–33° with shaking (Note 1) when the optical density (Note 2) reaches about 1·5, the culture is transferred to fresh medium containing 25 μg. $FeSO_4.7H_2O$ per 100 ml. and 4 ml. of 50 per cent maltose solution per 100 ml. The amount of culture transferred is adjusted so that exponential growth will cease at a convenient time. Ordinarily an O.D. of about 0·04 is suitable. The culture is then grown at 32–33° with shaking and will continue to grow exponentially for 15–16 hr. until an O.D. of about 2·5 is reached (Note 3).

Step 2.—The culture grown in Step 1 is harvested by centrifugation in the cold. The cells are then washed twice with iron-free medium (Note 4) and used as inoculum for Step 3.

Step 3.—The washed packed cells are suspended as evenly as possible in a small volume of iron-free medium and then made up to optical density of approximately 5·0 in iron-free medium containing 8 ml. of 50 per cent maltose solution per 100 ml. (Note 5). Finally, the heavy suspension is placed on the shaking machine and incubated at 32–33° with shaking (Note 6). The culture enters the declining growth phase immediately with liberation of toxin, as illustrated

by the accompanying Table. Growth and toxin production are complete in about 18–20 hr.

Note 1.—We have used a rotating shaking machine, usually adjusted to 240 r.p.m. We have also used a shaking shelf with success.

Note 2.—Optical density (O.D.) is determined at 590 mμ on suitable dilutions in distilled water using a Bausch and Lomb spectrophotometer with 1 cm. light path. Some difficulty may be encountered in obtaining a homogeneous cell suspension for turbidity measurement after the centrifugation of Step 2. This difficulty may be overcome by use of a Potter-Elvejhem type homogenizer.

Note 3.—The division time of the SM1 strain in AMC medium was found to be 150–160 min. at 33–34°. The time of harvesting is the most crucial factor during Step 1 if good yields of toxin are to be obtained in Step 3. Thus, cells should be harvested just after the end of the exponential growth phase at a time when small amounts of toxin (1 or 2 Lf per ml.) have already appeared in the medium. If the cells are collected too soon, toxin production is delayed and the final yield will be low. If organisms in Step 1 are allowed to continue growth for too long at a declining rate, poor growth will be obtained in Step 3 and again this will cause poor yields of toxin.

Note 4.—Centrifugation and washing are carried out under sterile precautions in acid-cleaned glassware. Sterility is not a critical factor at this stage, however, because of the large inoculum used in Step 3 and because toxin production only occurs over a limited period of time.

Note 5.—The beneficial effect of maltose on toxin production has never been satisfactorily explained. Excellent growth is obtained in Step 3 when maltose is replaced by glucose but no significant amounts of toxin or indeed of extracellular protein are liberated.

Note 6.—The volume of medium per flask, the rate of shaking, and the concentration of maltose, all influence the availability of oxygen and affect the pH during the growth period. These three factors are of importance in determining the final yield of toxin. The rate of shaking and volume must be adjusted according to the size of flask used. The results shown in the Table were obtained using 40 ml. of medium in 300 ml. Erlenmeyer flasks on a shaking machine rotating 240 times per min.

Purification of Toxin Produced in Shake Culture

Since toxin comprises 70–75 per cent of the total protein released by the SM1 strain under the conditions described above, highly purified preparations of toxin can be isolated in good yield by a simple and rapid procedure as illustrated by the following example. Two hundred ml. of culture supernate containing a total of 15,000 Lf of toxin and 53·6 mg. total TCA-precipitable protein were dialysed in the cold against 800 ml. saturated ammonium sulphate containing 0·02 M phosphate buffer and adjusted to pH 7 with ammonia. The precipitate was collected the following day by centrifugation and redissolved in 25 ml. 0·02 M phosphate. The fraction precipitating between 45 and 55 per cent saturation with ammonium sulphate was then collected, dissolved in buffer and dialysed against 0·02 M phosphate pH 7 until free from sulphate. The dialysed solution measured 7 ml. and contained a total of 9800 Lf and 26·6 mg. TCA-precipitable protein.

The yield of toxin was therefore 65 per cent and its specific activity was 2·72 μg. protein per Lf corresponding to a purity of about 90 per cent (see Pappen-

heimer and Yoneda, 1957). Other preparations fractionated by the same procedure showed a single homogeneous peak when examined in the Tiselius electrophoresis apparatus.

SUMMARY

A method has been described for production of diphtheria toxin in high yield in shaken cultures in a simple medium containing only 3 amino acids, maltose, vitamins and salts. Since toxin comprises up to 75 per cent of the total TCA-precipitable extracellular protein, the method permits rapid isolation of toxin of a high grade of purity. The method has also proved useful in the preparation of purified ^{35}S-L-methionine and ^{35}S-L-cystine-labelled toxin and in studies on the kinetics of toxin formation.

TABLE.—*Growth and Toxin Production by SM1 Strain in AMC Medium*

Time (hr.)	Growth O.D. 590 mμ.	Toxin* Lf/ml.
0	4·9	0
1	7·05	4
2	8·7	10
3	9·5	15
4	10·5	23
6	12·0	40
9	14·0	70
11	14·75	95
21	21·00	150

* At 11 hr. the total TCA-precipitable protein was 321 μg/ml. Using the factor 2·44 μg. toxin per Lf (Pappenheimer and Yoneda, 1957) it may be calculated that toxin comprised nearly 75 per cent of the total protein released.

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A REINVESTIGATION OF THE DIPHTHERIA TOXIN-ANTITOXIN FLOCCULATION REACTION USING ^{35}S-METHIONINE-LABELLED TOXIN*

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THE reaction between antibodies produced by the horse and their corresponding protein antigens is often characterized by a broad region (so-called equivalence zone) in which precipitation of both antigen and antibody is almost complete. The system which has received the most detailed quantitative study is the flocculation reaction between diphtheria toxin and antitoxin (Marrack and Smith, 1930; Healey and Pinfield, 1935; Pappenheimer and Robinson, 1937). Pappenheimer and Robinson showed that for certain horse antitoxins which they examined, precipitation of both toxin and antitoxin was virtually complete over a wide range as determined by sensitive tests for excess toxin and antitoxin in the supernates. When the amount of antitoxin was kept constant, addition of increasing amounts of toxin gave a linear curve within the zone of equivalence when plotted against the total nitrogen specifically precipitated. The slope of this straight line was equal to the ratio of toxin nitrogen specifically precipitated to total toxin nitrogen or Lf units added and thus gave a measure of "immunological" purity for the toxin preparation used.

Cohn and Pappenheimer (1949) found that the immunological purity of a given toxin preparation (*i.e.*, per cent specifically precipitable by antitoxin) was independent of the species serving as a source of antitoxin. They found that the reaction of toxin with rabbit, guinea-pig, monkey and human antitoxins gave the same type of characteristic quantitative precipitin curve as found for other protein-antiprotein systems in the classic studies of Heidelberger and Kendall (1935). Using the figure for immunological purity calculated for a given toxin preparation from its reaction with horse antitoxin, precipitin curves with human antitoxin and the same toxin obeyed almost exactly the theoretical equations developed by Heidelberger and Kendall (1935) for a single antigen-antibody system.

In a series of papers, Pope and his collaborators (Pope, Stevens, Caspary and Fenton, 1951; Pope and Stevens, 1953*a*, *b*; Pope, 1954) have sharply criticized the conclusions drawn by Pappenheimer and Robinson (1937) and by Pappenheimer (1942) on the grounds that the quantitative flocculation curve is not truly linear, but rather consists of a series of overlapping bell-shaped curves, each due to a different antigen-antibody system. Using concentrated solutions of toxin

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and antitoxin incorporated in gels, Pope *et al.* (1951) have shown many lines of precipitation (up to 24), each of which they feel may be attributed to separate non-toxin antigen-antibody systems. The views of Pope and his co-workers appear to have been accepted by recent reviewers (Oakley, 1954; Marrack, 1955).

The conclusions of Pappenheimer and Robinson have also been criticized by Burtin (1955) on the ground that the methods which they used for detecting excess toxin and antitoxin would have failed to detect soluble toxin-antitoxin complexes. In quantitative studies using ^{131}I-labelled human serum albumin and its corresponding horse antibody, Burtin found that as much as 27 per cent of the radioactivity remained unprecipitated in supernates from specific precipitates formed within the equivalence zone.

If the precipitate formed in the toxin-horse antitoxin reaction were indeed composed largely of other antigen-antibody systems as claimed by Pope (1954) or if precipitation were incomplete (Burtin, 1955), it is clear that the deductions made by Pappenheimer and Robinson (1937), Pappenheimer (1942), Cohn (1952) and others would be of doubtful significance. Moreover, serious doubt would be cast also on the interpretation of quantitative results obtained with other antigen-antibody systems using antibody prepared in other species.

We have, therefore, undertaken a reinvestigation of the specificity and completeness of precipitation of diphtheria toxin by horse antitoxin using purified toxin, labelled internally with ^{35}S-L-methionine.

MATERIALS AND METHODS

^{35}S-L-methionine and ^{35}S-L-cystine were obtained from Abbott Laboratories, Chicago, Illinois.

Radioactivity was measured with a thin-window Geiger-Mueller counter. Samples were sufficiently thin so that there was no appreciable self-absorption.

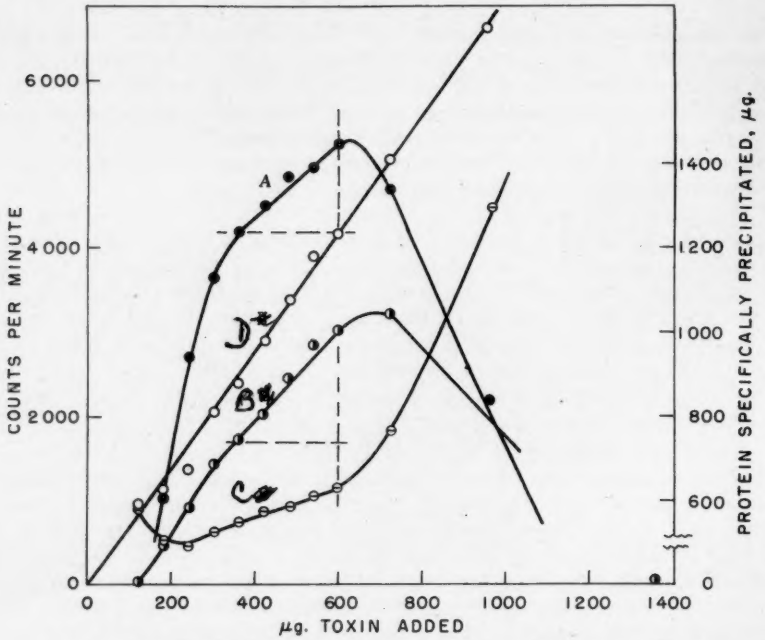
Preparation of ^{35}S-methionine toxin

^{35}S-toxin was prepared by the method described in the preceding paper (Yoneda, 1957). ^{35}S-L-methionine diluted 1/5 in unlabelled L-methionine was used. The total methionine content of the inoculum medium was 30 μg./ml., of the final medium, 150 μg./ml. A small portion of the latter was stored in the frozen state and counted at the time when the immunological experiments were carried out. The specific radioactivity varied in different experiments between 500 and 1400 counts per min. per μg. methionine.

Preparation 1

A volume of 147 ml. of culture supernate containing a total of 14,000 Lf ^{35}S-toxin was dialysed for 24 hr. in the cold against 1000 ml. of saturated ammonium sulphate. The precipitate was collected by centrifugation and dissolved in 20 ml. 0·02 M phosphate buffer, pH 7·0. This solution was clarified by centrifugation at 5000 r.p.m. in the cold and the supernate dialysed against 180 ml. 50 per cent saturated $(NH_4)_2SO_4$ to give 0·45 saturation at equilibrium. The precipitate (Fraction 1) was dissolved in phosphate buffer, dialysed against the same buffer to remove sulphate and made up to 20 ml. This fraction contained 4·7 mg. TCA-precipitable protein (14 per cent of total protein recovered) and less than 20 Lf per ml. of ^{35}S-toxin. The supernate (17 ml.) from Fraction 1, was dialysed against 170 ml. 61 per cent saturated ammonium sulphate. The precipitate (Fraction 2) was made up to 20 ml. after dialysis. It contained 8500 Lf ^{35}S-toxin and 24·2 mg. protein corresponding to an immunological purity of 86 per cent. Fraction 2 was used for determining the methionine content of toxin (Table I) and in carrying out the quantitative flocculation reaction (Figure). The supernate from Fraction 2 (15 ml.) was dialysed against 150 ml. 76 per cent saturated ammonium sulphate. The precipitate (Fraction 3) was collected and made up

to 10 ml. after dialysis against phosphate buffer. The supernate was discarded: 1400 Lf and 4·4 mg. TCA-precipitable protein were recovered in Fraction 3. Thus 70 per cent of the total ^{35}S-toxin was recovered in Fractions 2 + 3 and the remaining 30 per cent was unaccounted for.



FIGURE—Quantitative flocculation of purified ^{35}S-toxin by antitoxin 5353 AD.

A.—Total specifically precipitable protein.
B.—Specifically precipitable ^{35}S-methionine.
C.—^{35}S remaining in supernate.
D.—Total radioactivity recovered, B + C.

The region from which the slopes were calculated for curves A and B is indicated by dashed lines.

Preparation 2

A volume of 420 ml. of culture supernate containing 36,000 Lf ^{35}S-toxin was dialysed against 1600 ml. saturated ammonium sulphate solution. The precipitate was collected and dissolved in 30 ml. phosphate buffer. Six ammonium sulphate fractions were then prepared: F1, 0–0·4; F2, 0·4–0·5; F3, 0·5–0·55; F4, 0·55–0·6; F5, 0·6–0·65; F6, 0·65–0·75 saturation. The bulk of the toxin and protein (*ca.* 80 per cent of total recovered) was contained in Fractions 2 and 3. No detectable toxin and small amounts of protein were present in Fractions 1 and 6. The immunological purity of F2, 3 and 4 was 91–94 per cent as shown in Table I.

^{35}S-L-methionine P-proteins

The inoculum was grown in shake culture containing 4 μg./ml. $FeSO_4.7H_2O$ and 30μg./ml. ^{35}S-L-methionine. The final culture contained 4 μg/.ml. ferrous sulphate and 150 μg./ml. ^{35}S-methionine (specific activity 1320 counts/min./μg. methionine) and was harvested after 30 hr. incubation at 32–33° in the shaking machine. The culture supernate (60 ml.) was dialysed against 440 ml. saturated ammonium sulphate. The precipitate was collected and

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In the Figure, p. 196, the designations of the curves should read, from above downwards, A, D, B, C.

dissolved in 20 ml. 0·02 M phosphate buffer, pH 7·0. This solution was further fractionated and the 0·3–0·7 saturated ammonium sulphate precipitate collected, dialysed to remove sulphate and made up to 4 ml. in phosphate buffer. The yield was 1·47 mg. ^{35}S-P-proteins per ml. with a specific activity of 14·9 counts/min./μg. protein, corresponding to 1·14 per cent methionine. The solution contained about 5 per cent nucleic acid. Toxin could not be detected by flocculation but guinea-pig titration showed about 1 L+ dose per ml.

A crude preparation of ^{35}S-P-proteins was also prepared by growing the SM strain as a pellicle on AMC medium containing 4 μg. ferrous sulphate and 40 μg. ^{35}S-methionine/ml. The culture was harvested after 7 days' incubation at 32–33°. After removing the bacteria by high-speed centrifugation, the supernate contained 194 μg. TCA-precipitable protein/ml. The total radioactivity was 4435 counts/min./ml. of which 1315 were precipitated by TCA.

Antitoxin

Antitoxin, containing 500 flocculating units/ml., was kindly supplied by the Biologic Laboratories, Massachusetts Department of Health. Antitoxin 5353 AD was a purified gamma globulin preparation containing 444 units/ml., previously shown in three laboratories to give a single broad zone of flocculation (Pappenheimer, Edsall, Lawrence and Banton, 1950).

Protein determinations were carried out by the method of Lowry, Rosebrough, Farr and Randall (1951), standardized by micro-Kjeldhal determination of nitrogen using a highly purified toxin solution. Specifically precipitable protein was determined in 0·25-N acetic acid at 277 mμ in the Beckmann spectrophotometer (Gitlin, 1949).

EXPERIMENTAL

Methionine and Cystine Contents of Diphtheria Toxin

^{35}S-L-methionine toxin was prepared and purified by ammonium sulphate fractionation and dialysis as outlined above. For determination of methionine content, a known number of Lf of labelled toxin were allowed to react with an equivalent number of units of horse antitoxin for 1 hr. at 37°. The precipitate was left in the cold overnight and then centrifuged and washed with cold saline until no significant radioactivity could be detected in the supernate. The washed precipitate was dissolved and made up to 2 ml. in 0·25-N acetic acid and 0·1 ml. samples used for determination of radioactive counts. The weight of methionine specifically precipitated was then calculated from the specific activity of the ^{35}S-L-methionine which had been added to the original culture medium. The latter was counted at the same time as the specific precipitate so that no correction was necessary for ^{35}S decay. The Lf units of toxin specifically precipitated were converted to μg. protein using the factor 2·44 μg./Lf. From columns 2 and 3 of Table I, it can be seen that the ^{35}S-L-methionine specifically precipitated per Lf from preparation 1 was the same, within experimental error, whether from the original crude culture filtrate or from purified ammonium sulphate fractions. Moreover, the methionine specifically precipitated per Lf unit and therefore the methionine content of the precipitated toxin (column 7) is independent of the per cent of the total radioactivity which is specifically precipitable (column 10). Almost the same methionine content for the toxin protein was calculated from the slope of the quantitative flocculation curve described below. Table I shows that for both preparations, the methionine content of toxin, as determined by specific precipitation with antitoxin (column 7), was lower than the methionine content of the total protein (column 8). In both preparations 1 and 2, the discrepancy was most pronounced in those fractions which showed the greatest solubility in ammonium sulphate. This finding suggests the presence of a small amount of protein impurity of high methionine content. Table I clearly shows

TABLE I.—*Methionine Content of Diphtheria Toxin and Specificity of the Toxin-Antitoxin Reaction*

(1) Ammonium sulphate fraction saturation	(2) Total toxin added (Lf)	(3) Specifically precipitable toxin* (μg.)	4) Specifically precipitable methionine† (counts/min.)	(5) TCA precipitable protein (μg.)	(6) TCA precipitable methionine (counts/min.)	(7) Methionine content of toxin (per cent)	(8) Methionine content of total protein (per cent)	(9) Specific precipitability: Protein‡ (per cent)	(10) Specific precipitability: Methionine§ (per cent)
Preparation 1									
Crude filtrate	190	464	3,150	—	5,400	1·39	—	—	—
0·45–0·60	170	415	2,780	504	3,560	1·37	1·45	83	77
0·60–0·75	210	513	3,200	687	8,445	1·27	2·5	75	38
0·45–0·60 (from Figure)	(96)	210‖	1,290‖	240	1,765	1·26‖	1·43	87·5	—
Preparation 2									
0·40–0·50	157	383	7,330	410	8,580	1·60	1·74	93·5	84·5
	220	537	10,030	573	12,000	1·55	1·74	94	83·5
0·50–0·55	150	366	6,480	402	7,800	1·48	1·62	91	83·5
	210	513	9,240	563	10,900	1·50	1·62	91	84·5
0·55–0·60	160	391	7,600	422	11,800	1·63	2·33	92	64
0·60–0·65	150	366	6,800	—	13,600	1·54	—	—	50
0·65–0·75	—	—	—	260	8,000	—	2·57	—	—

* Calculated assuming 2·44 μg. protein per Lf unit.
† The specific activity of ^{35}S-L-methionine used in preparation 1 was 490 and in preparation 2 was 1,200 counts per μg. per minute.
‡ From columns (3) and (5).
§ From columns (4) and (6).
‖ Calculated from slopes (Figure).

that the diphtheria toxin protein contains methionine. While a high degree of accuracy cannot be claimed, the methionine content is estimated as 1·45 ± 0·15 per cent.

Using the same method, and toxin prepared on ^{35}S-L-cystine-containing culture medium, the presence of cystine, specifically precipitated by antitoxin, has also been demonstrated. The results are not reported in detail since the experiments were carried out before a gas-flow counter became available. A cystine content of about 0·6 per cent was found, a figure which is probably 30–40 per cent low.

Specificity of Toxin-antitoxin Flocculation

Experiment 1

To each of two Pyrex centrifuge tubes was added 408 Lf partially purified unlabelled toxin in a volume of 1·45 ml. To tube A was added 10 ml. of crude culture filtrate containing 194 μg. TCA-precipitable ^{35}S-P-proteins per ml. with a specific activity of 6·8 counts/μg. As seen from Table II, the total counts

TABLE II.—*Specificity of Toxin-antitoxin Reaction*

A. 408 Lf unlabelled toxin + 430 units antitoxin + 10 ml. high-iron ^{35}S-methionine culture filtrate.
B. 408 Lf unlabelled toxin + 430 units antitoxin + 10 ml. uninoculated ^{35}S-methionine-containing culture medium.

Tube	Unlabelled toxin specifically precipitated (μg.)	Culture filtrate containing ^{35}S-P-protein: Total protein (μg.)	Total methionine (counts/min.)	TCA precipitable methionine (counts/min.)	Total radioactivity in specific precipitate (counts/min.)	Maximum co-precipitable P-protein (μg.)
A	995	1,940	44,350	13,150	30	4·4
B	995	0	176,000	0	40	—

added were 44,350 per min. To tube B was added 10 ml. of uninoculated ^{35}S-methionine-containing AMC medium, with a total of 176,000 counts per min.: 1 ml. of antitoxin 5353 AD was then added to each tube with thorough mixing. The mixtures were allowed to flocculate 1 hr. at 37° and then placed in the cold overnight. The precipitate was collected and washed 3 times with 5 ml. changes of AMC medium containing 1 mg./ml. unlabelled methionine. The precipitate was dissolved in 0·1 N-NaOH and made up to 2 ml. As seen in Table II, only 30 counts per min. out of a total of 13,150 ^{35}S-P-protein counts per min. were found in the specific precipitate, equivalent to only 0·4 per cent of the toxin protein added. It will be noted that 40 counts per min. were precipitated in the control tube.

Experiment 2

^{35}S-P-proteins were prepared as described in the section on methods. The 0·6 saturated ammonium sulphate fraction, after removal of sulphate by dialysis, contained 1·47 mg. TCA-precipitable protein per ml., and 21,800 counts per ml. To each of 2 Pyrex centrifuge tubes was added 0·5 ml. of ^{35}S-P-protein (735 μg.). To tube A was added 162·5 Lf purified unlabelled diphtheria toxin diluted

to 4 ml. in broth and 0·4 ml. antitoxin 5353 AD (178 units). To tube B was added 3·4 ml. of broth containing 200 μg. crystalline ovalbumin and 1 ml. rabbit anti-ovalbumin serum. Both tubes were allowed to flocculate at 37° for 1 hr. and were then placed in the cold overnight. The precipitates were centrifuged, washed 3 times with 3 ml. changes of chilled saline and finally each precipitate was dissolved in 0·25-N acetic acid and made up to 2 ml. From the absorption at 277 mμ, it was calculated that tube A contained a total of 1·50 mg. and tube B, 1·28 mg. specific precipitate. As seen from Table III, only a total of 90 out of

TABLE III.—*Specificity of Toxin-antitoxin Reaction*

Tube A. 162 Lf unlabelled toxin + 178 units antitoxin + ^{35}S-P-protein.
Tube B. 200 μg. ovalbumin + 1 ml. rabbit antiovalbumin + ^{35}S-P-protein.
Total volume 4·9 ml.

Tube	Unlabelled antigen specifically precipitated (μg.)	^{35}S-P-protein added (μg.)	^{35}S-P-protein added (counts/min.)	Radioactivity in specific precipitate (counts/min.)	Maximum co-precipitable P-protein (μg.)
A	395	735	10,900	90	6·1
B	200	735	10,900	0	0

10,900 counts per min. of ^{35}S-labelled P-protein were found in specific precipitate A and the control was entirely negative. Thus a maximum of 6·1 μg. of P-protein could have been carried down with the 1·5 mg. toxin-antitoxin complex or only 1·5 per cent of the toxic protein added. However, it is probable that even this small percentage can be largely accounted for by co-precipitation of the trace of labelled toxin (about 1 L+ dose) which contaminated the P-protein preparation added.

Quantitative flocculation reaction

To a series of 12 clean Pyrex centrifuge tubes were added increasing amounts (from 47–533 Lf) of ^{35}S-L-methionine-labelled purified toxin and saline to bring the volume to 2 ml. The toxin (Preparation 1, Fraction II) contained 2·44 μg.* protein per Lf and 7·0 counts/μg. protein. One ml. of diluted antitoxin 5353 AD (148 units) was added with thorough mixing to each tube. The mixtures were placed in the water bath at 37° for 2 hr. and then in the cold overnight. The precipitates were centrifuged and the supernates saved for radioactive count. The precipitates were washed twice with 3 ml. of cold saline and then dissolved and brought to 2 ml. in 0·25 N acetic acid. Protein was determined by absorption at 277 mμ (Gitlin, 1949) and radioactivity determined in the gas-flow counter. The Figure summarizes the results obtained. Between 360 μg. and 600 μg. of total protein added, the specifically precipitable protein (curve A) and specifically precipitable radioactive counts (curve B) show a linear increase. From the slope of curve A, it can be calculated that 87·5 per cent of the protein added was specifically precipitated by antitoxin, a value which compares favourably with 83 per cent calculated from the specific activity. Seventy-seven per cent of the ^{35}S was specifically precipitated over the same region of equivalence. The increases in curves A and B over this region correspond to 6·15 counts/μg. speci-

* By absorption at 277 mμ, using the factor for gamma globulin given by Gitlin (1949); 2·74 μg. protein per Lf were found by the method of Lowry *et al.* (1951).

fically precipitable toxin protein, which is equivalent to a methionine content of 1·26 per cent (see Table I).

DISCUSSION

In their study of the flocculation reaction between diphtheria toxin and horse antitoxin, Pappenheimer and Robinson (1937) selected for detailed analysis antitoxins whose quantitative behaviour with toxin approximated as closely as possible to what they regarded as the " ideal system ". In other words, with the particular sera which they used, precipitation of both toxin and antitoxin was virtually complete over a broad equivalence zone and antibodies other than antitoxin, if present, did not contribute significantly to the toxin-antitoxin precipitate. Other antitoxic and anti-protein horse antisera often show deviations from the " ideal " and occasionally these deviations may be considerable. It is probably owing to the failure of the early workers to appreciate and emphasize the frequency and extent of the variation from one serum to another, that much of the subsequent confusion regarding the quantitative behaviour of horse antibody has arisen.

Before discussing further the discrepant findings of certain workers, it seems worth while to summarize in some detail the evidence that the precipitation of diphtheria toxin by a *suitably chosen* horse antitoxin is specific and complete over a broad equivalence zone.

The present studies have shown that when purified unlabelled toxin is allowed to flocculate with antitoxin in the presence of excess non-toxin diphtherial proteins labelled with ^{35}S-methionine, no appreciable radioactivity is carried down with the specific precipitate. This finding is contrary to what one would expect if antibodies against non-toxin proteins contaminated the toxin-antitoxin specific precipitate as repeatedly claimed by Pope and his co-workers.

When ^{35}S-methionine-labelled toxin is allowed to flocculate with antitoxin within the equivalence zone, the same amount of radioactivity is precipitated per Lf unit irrespective of the purity of the toxin fraction used. This finding confirms the earlier observations of Pappenheimer and Robinson (1937) and others who showed that the specifically precipitable toxin (or toxoid) nitrogen was the same whether crude or purified toxin (or toxoid) was used as antigen.

Many antitoxins show a broad region in which the total specifically precipitable nitrogen increases linearly with increased toxin (or toxoid) added (Robinson and Pappenheimer, 1937 ; Cohn and Pappenheimer, 1949 ; Lepow and Pillemer, 1952). The slope of the linear portion of the curve is almost the same as the specific activity of the toxin employed when highly purified toxin is used as antigen. This fact indicates that all of the toxin and antitoxin are precipitated and that the increased nitrogen is due solely to toxin added within this region. This conclusion is confirmed by the present studies which show a linear increase in ^{35}S-methionine precipitated within the equivalence zone. Certain other protein-horse anti-protein systems, such as the scarlet fever toxin-antitoxin (Hottle and Pappenheimer, 1941) and the conalbumin-anti-conalbumin systems (Cohn, Wetter and Deutsch, 1949) have shown similar quantitative behaviour.

When concentrated solutions of antitoxin and toxin are allowed to interact in a gel, multiple zones of precipitate often form, each apparently due to a different antigen-antibody system (Pope *et al.*, 1951). Bowen (1952) has shown that the

number of lines observed increases according to the length of time of immunization. Short term immunization may yield antitoxins which show only a single line of precipitate with purified toxin. Bowen showed that when antitoxins showing many minor lines of precipitation are appropriately absorbed with purified toxin, the major line corresponding to toxin-antitoxin is removed leaving the minor lines intact. Finally, Bowen showed that while the reaction of a concentrated antitoxin with concentrated toxin may give rise to many lines by the gel-diffusion technique, relatively slight dilution causes all but the toxin-antitoxin line to disappear. Since the line of toxin-antitoxin precipitate persisted to high dilutions in Bowen's experiments, it was clear that other antigens accounted for only a small fraction of the total antigenic material present in his toxin preparation.

In many human antitoxins and in suitably absorbed rabbit antitoxin, the point of neutralization of toxin coincides exactly with the point of maximal specific precipitation. Such antitoxins show only a single band when examined by the Oudin technique (Oudin, personal communication). The immunological purity of a given toxin as estimated from its quantitative precipitin reaction with human, monkey, rabbit or guinea-pig antitoxin, is the same as that calculated from the slope of its flocculation reaction with horse antitoxin (Cohn and Pappenheimer, 1949).

In the present experiments, at least 85 per cent of ^{35}S-methionine-labelled toxin (Preparation 2) was specifically precipitated by the antitoxin used as determined by radioactive counts. Since the impurities in the toxin preparation had a higher methionine content, it is estimated that the amount of toxin remaining unprecipitated as a soluble complex could not have exceeded 5–10 per cent.

The high dissociability of the scarlet fever toxin-antitoxin complex in dilute solution made it possible to calculate the solubility of that system with some accuracy. It was shown that at the relatively high concentration of reagents studied, precipitation of scarlet fever toxin at the flocculation point was at least 98 per cent complete (Hottle and Pappenheimer, 1941).

The careful experiments of Bowen, Polley and Huang (1954) on the amount of nitrogen specifically precipitated in the equivalence zone using varying concentrations of diphtheria toxin and antitoxin show that solubility of the specific precipitate is minimal at the flocculation point and increases as one proceeds in either direction away from this point. At the usual concentrations of antitoxin (60–100 units/ml.) at which quantitative determinations are carried out, the data of Bowen *et al.* indicate that precipitation may be *at least* 95 per cent complete throughout most of the equivalence zone. Dr. Marcel Raynaud (personal communication) has recently carried out quantitative studies at the Pasteur Institute using highly purified ^{131}I-labelled diphtheria toxin and various horse antitoxins. Within the zone of equivalence, he recovered 92–97 per cent of the radioactivity in the specific precipitate.

The specificity and completeness of precipitation of the toxin-antitoxin complex were also demonstrated by the ultracentrifugal studies of Pappenheimer, Lundgren and Williams (1940). They used toxin and antitoxic globulin, both of which were homogeneous by sedimentation, electrophoresis and by diffusion. The supernate obtained after allowing the reagents to precipitate at the flocculation point showed no trace of the toxin component, indicating its complete removal, nor could any evidence for a soluble toxin-antitoxin complex be obtained. Such a complex would have been easily detected if present in significant concentration.

Most of the evidence summarized above existed before the papers by Pope and his co-workers appeared claiming that the toxin-antitoxin flocculation reaction was not specific and that the purest preparations of toxin isolated by other workers were grossly contaminated with proteins unrelated to toxin. The purest preparations of diphtheria toxin isolated by Eaton (1936), Pappenheimer (1937) and Agner (1950) and of toxoid by Pillemer, Toll and Badger (1947) showed a specific activity approaching 2200 $\pm$ 10 per cent Lf/mg. N. The preparations satisfied all of the usual criteria of purity for proteins. They were homogeneous by sedimentation, electrophoresis and by diffusion and in the case of toxoid, the preparation of Pillemer *et al.* showed constant solubility. In recent years toxin preparations of higher specific activity have been isolated. The toxin of Lepow and Pillemer (1952) contained nearly 2600 Lf/mg. N. Pope and Stevens (1953*c*) and Norlin (1955) obtained toxin with more than 3000 Lf/mg. N. Preparation 2 used in the present experiments had a specific activity of almost 2400 Lf/mg. N, and it was estimated to be only 90 per cent pure from the immunological data. It is important to recognize that *the number of Lf per mg./N is a measure of specific activity and not of purity.* Whereas nitrogen can be determined with an accuracy of 0·5 per cent or better, the flocculation unit is a relative value referred to a standard antitoxin. It is doubtful whether Lf values are significant to better than 5 or 10 per cent. As pointed out by Jerne (1951) and by Maaloe and Jerne (1952), antitoxins vary greatly in their affinity for toxin and in other properties from one animal to another and vary in a given animal during the course of immunization. Thus estimations of purity based on specific activity must be viewed with scepticism.

We must also distinguish between chemical and immunological purity. It has been known since the time of Ehrlich that diphtheria toxin is not a homogeneous substance since it may lose toxicity without loss of its capacity to react with antitoxin. Depending on the extent of spontaneous toxoid formation, toxin may vary in solubility and electrophoretic behaviour (Agner, 1950). Formol toxoid has a different solubility and isoelectric point from toxin (Pillemer *et al.*, 1947), yet it is indistinguishable immunochemically from toxin. It is therefore convenient to measure " immunological " purity which we may define as the per cent of a given toxin preparation specifically precipitable by a suitably chosen antitoxin.

We have emphasized the importance of choosing an appropriate antitoxin for quantitative study of the toxin-antitoxin reaction. In our experience, diphtheria antitoxins which show more than one zone of flocculation are uncommon, and when other zones do occur they can usually be distinguished from the zone of toxin flocculation without difficulty. Occasionally, however, as seems to have been the case with the antilecithinase (*Clostridium welchii*) studied by van Heyningen and Bidwell (1948), overlapping zones of flocculation may occur. Obviously, such sera are unsuitable for quantitative work on the toxin-antitoxin reaction itself, although they may be useful for other purposes.

Antitoxins may show variability and inhomogeneity in other ways. The classic studies of Glenny and Barr (1932) and more recently by Jerne (1951) show that there are large differences in " avidity " or " dilution ratio " from one sample to another taken during the course of immunization or from one animal serum to another. Possibly as a reflection of these differences in avidity, quantitative study shows that the extent of the zone of complete precipitation varies

not only with the dilution at which the reaction is carried out (Bowen *et al.*, 1954) but also varies at a given concentration of antitoxin from one serum to another. In some horse antitoxic sera the linear region in which precipitation of antigen and antibody is complete is narrow or absent, particularly if the reaction is carried out with relatively dilute reagents. Using ^{131}I-labelled human serum albumin and its corresponding horse antibody, Burtin (1955) found that 17–28 per cent of the antigen-antibody complex remained unprecipitated even at the flocculation point. He attributed this solubility to the high content of " incomplete " or " univalent " antibody in his serum. As an extreme instance of incomplete precipitation, we may cite the example of the horse immunized against ovalbumin by Pappenheimer (1940). An early bleeding from this animal contained appreciable amounts of antibody which reacted with ovalbumin but gave no precipitate at all.

In order to determine the purity of an antigen such as diphtheria toxin by the immunochemical method, it is clearly essential to select a serum giving almost complete precipitation over as wide a range of toxin-antitoxin ratios as possible. Bowen *et al.* (1954) showed that solubility of the complex increases as one leaves the flocculation point and approaches either end of the equivalence zone. Their experiments demonstrate that as toxin and antitoxin are allowed to react in more dilute solution, the curves of precipitation become increasingly rounded in shape and the linear portion disappears. In the particular serum which they chose for detailed study, the curve became almost bell-shaped when antitoxin was diluted to a concentration of 20 units per ml. Pope and Stevens (1953*a*) have suggested that the true curve of precipitation for toxin-antitoxin is symmetrical and they too have obtained bell-shaped curves. Pope and Stevens carried out the reaction at a constant antitoxin concentration of 20 units per ml.

It was shown by Pope (1938, 1939) that short treatment of horse antitoxin with a proteolytic enzyme yields an antitoxin of higher specific activity which is no longer coagulable on heating to 58° at pH 4·2. Petermann and Pappenheimer (1941) and Rothen (1942) showed that exposure to pepsin or trypsin causes a splitting off of an inactive fragment of the globulin molecule which may be removed by coagulation at 58° at pH 4·2. The antitoxin groups remain in solution attached to a smaller molecule of nearly doubled specific activity. When exposure to the enzyme is brief, the product flocculates with toxin more rapidly than before digestion. Although less total nitrogen is precipitated in the flocculation reaction, the extent of the zone of complete precipitation remains unchanged. Recently, by repeated alternation of specific precipitation followed by dissociation by means of pepsin, Pope and Stevens (1953*a*) have isolated an antitoxin of very high specific activity per mg./nitrogen. Some of the preparations failed to give any precipitate with toxin although still capable of neutralizing its toxicity. The sedimentation behaviour of antitoxin prepared by such repeated exposure to proteolysis has not been examined but its high specific activity suggests a relatively small molecule, perhaps of lower molecular weight than toxin. In any event, it may possibly be of significance that it is with antitoxin prepared in this manner that Pope and Stevens (1953*a*) obtained their symmetrical curves with toxin.

Symmetrical curves were also obtained by Pope and Stevens (1953*b*) with antitoxin which had been subjected to an unusual series of absorptions and fractionation procedures. After exhaustive absorption with a heavy suspension

of washed diphtheria bacilli, followed by absorptions with antigens contained in a culture filtrate from organisms grown in excess iron, the antitoxin was treated with excess of "peptic toxin". Pope and Stevens (1953*a*) showed that after treatment of toxin with pepsin, a product was obtained which failed to neutralize antitoxin (presumably by *in vivo* test). The immunological properties of peptic toxin were puzzling. The product gave a direct flocculation at a value almost the same as that of the original toxin but showed negligible toxin by flocculation test when blended with untreated toxin, a curious discrepancy for which the authors gave no satisfactory explanation. After adding excess peptic toxin to previously absorbed antitoxin, Pope and Stevens fractionated with ammonium sulphate "to separate antigen-antibody complexes". After the final treatment with peptic toxin, followed by salt fractionation, the shape of the flocculation curve became symmetrical and the total amount of precipitate decreased. In some instances almost no antitoxin precipitated after treatment. In our opinion the simplest explanation for the interesting results obtained by Pope and Stevens is that peptic toxin does contain material which is still able to form soluble complexes with bivalent antitoxin. These complexes are removed during ammonium sulphate fractionation and the antitoxin which remains consists mostly of incomplete or "univalent" antitoxin. In this connection the recent experiments of Katsura (1955) are of interest. In an addendum to his paper, Katsura reports that by fractional salting out of soluble toxin-antitoxin complexes, followed by dissociation with pepsin, he succeeded in isolating antitoxin fractions which varied greatly in immunochemical properties and *in vivo-in vitro* ratios.

The method for estimation of immunological purity of a protein by determining the slope of the linear portion of its flocculation curve with horse anti-protein requires that the amounts of specific precipitate formed be measured with a high degree of precision. Since our concern is with relatively slight *differences* in total protein, small errors in protein determination may lead to large errors in the calculation of per cent purity. In our opinion micro-Kjeldahl determination of nitrogen is the most satisfactory method available for precise determination of the protein precipitates. On the other hand it seems doubtful whether the turbidimetric method (Pope and Stevens, 1953*a*), is sufficiently precise for accurate determination of the slope. Nevertheless, the rapidity and ease with which turbidity may be measured make the method a useful one for many other purposes.

Before closing the discussion, some comment seems worth while regarding the sulphur-containing amino acids of diphtheria toxin. Pappenheimer (1937) reported that the sulphur content of the purified toxin protein was about 0·75 per cent. On the other hand, Pillemer *et al.* (1947) found no sulphur in purified toxoid by a variety of tests. The present study conclusively demonstrates the presence of both methionine and cystine in diphtheria toxin and confirms the findings of Ito, Uetake and Sasaki (1953) who obtained chromatographic evidence for the presence of both these sulphur-containing amino acids in purified toxin and toxoid. Woiwod and Linggood (1949) also showed the presence of methionine in purified toxin and toxoid by a chromatographic method.

SUMMARY

When unlabelled diphtheria toxin was allowed to flocculate with horse antitoxin in the presence of ^{35}S-L-methionine-labelled non-toxic-diphtherial proteins, no significant radioactivity was carried down with the specific precipitate. The

extreme specificity of the flocculation reaction of toxin with this antitoxin was further indicated by quantitative studies using ^{35}S-L-methionine-labelled toxin. These studies showed that the amount of methionine specifically precipitable by antitoxin was independent of the purity of the toxin preparation used. The methionine content of diphtheria toxin has been calculated to be $1{\cdot}45 \pm 0{\cdot}15$ per cent. Similarly the cystine content of ^{35}S-L-cystine-labelled toxin has been estimated to be at least 0·6 per cent.

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OBSERVATIONS ON THE DIPHTHERIA TOXIN-ANTITOXIN REACTION*

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PAPPENHEIMER AND YONEDA (1957) have reinvestigated the diphtheria toxin-antitoxin flocculation reaction using ^{35}S-methionine-labelled toxin and compared their results with those previously obtained by Pappenheimer and Robinson (1937). This new work, carried out by modern radio-active methods, appears to support the earlier observations of Pappenheimer and Robinson (1937) and to refute those of Pope and his colleagues (Pope, Stevens, Caspary and Fenton, 1951 ; Pope and Stevens, 1953*a*, *b*, *c* ; Pope, 1954*a*, *b*).

It is important in a problem of this kind to define the reacting systems very carefully : it is clear that the term " diphtheria toxin-antitoxin flocculation reaction " implies the reaction between the specific toxin, responsible for the lethal or dermonecrotic effects (or the toxoid produced from it), and the specific antibody that combines with this toxin. It is of interest to survey the materials with which the reaction is carried out.

Diphtheria Toxin

The methods of Eaton (1936*a*, *b*) and Pappenheimer (1937) applied to crude culture filtrates of *Corynebacterium diphtheriae* led to the isolation of a protein fraction with a purity of the order of 2100 Lf units per mg. of nitrogen. Following this isolation, the work of Pappenheimer and Robinson (1937) appeared to provide confirmatory evidence that this material was pure toxin, with a value of 2170 Lf units/mg. N. This value was widely accepted for many years. Pope *et al.* (1951) disproved this by obtaining toxin of higher value ; they isolated a small amount of material with a purity of 3200 Lf/mg. N. When they attempted to repeat the work of Pappenheimer and Robinson they failed to find any antitoxic sera of English or other origin behaving as did those reported by Pappenheimer and Robinson, and were therefore unable to use this method for the investigation of toxins, now available, with purities greater than were to be expected on immunochemical grounds. By the application of a gel-diffusion method based on that of Oakley and Fulthorpe (1953), Pope *et al.* (1951) were able to show that even the purest toxin preparations available contained a number of distinct antigens. This was true not only for their own preparations, but also for a toxin of a purity of 2170 Lf/mg. N prepared by Pappenheimer, which showed a total of 14 distinct lines in this test. The total amount of contaminating antigens was considered by Pappenheimer, Edsall, Lawrence and Banton (1950) to be of the order of 5 per

* The author of this paper was enabled to read the preceding paper in this issue by A. M. Pappenheimer and M. Yoneda before it went to press.—Ed.

cent for a toxin with a purity of 2170 Lf/mg. N. Bowen (1952) using the diffusion method confirmed the presence of many distinct antigens in purified toxins, but considered that the total amount of these other antigens did not substantially alter the figure of 0·00046 mg. N per Lf (2170 Lf/mg. N) found by Pappenheimer and Robinson (1937).

Other workers have, however, succeeded in isolating toxin of higher purity: Lepow and Pillemer (1952) reported a value of about 2600 Lf/mg. N and Norlin (1955) obtained toxin with more than 3000 Lf/mg. N. Raynaud and Relyveld (1956) have also obtained toxin of this order of purity.

Pope and Stevens (1953*c*) reported the isolation of a crystalline diphtheria-protein with both *in vivo* and *in vitro* properties of diphtheria toxin; this, after three crystallizations, gave what appeared to be a single line in the diffusion test and had a purity of 3200 Lf/mg. N. They expressed the view, after testing this material against various antitoxic sera, that it would be unwise to claim that this was the specific toxin in crystalline form.

Katsura (personal communication, 1957) has succeeded in isolating this crystalline protein and gives the purity as 3200–3400 Lf/mg. N. Raynaud and Relyveld (personal communication), following a visit to these laboratories, have also obtained a crystalline protein with similar purity. There is, therefore, independent support of the work of Pope and Stevens (1953*c*) that a crystalline protein with this high purity is obtainable, and that the contaminating antigens, present in earlier preparations, were there in much larger amounts than were indicated by Pappenheimer or Bowen.

Pope (1954*b*) in a study of the diphtheria antigen-antibody flocculation system used crystalline toxin-protein with two solvent systems, 0·9 per cent sodium chloride solution and 20 per cent ammonium sulphate. In saline a typical "flocculation reaction" was obtained, whereas in the other solvent there was a typical "precipitin-type" curve. By comparison, the amount of precipitate was always less in the "flocculation system" than in the "precipitin system". The ability of the crystalline toxin-protein to give varying amounts of "flocculate-precipitate", depending on the solvent system used, makes it clear that its reactions with antitoxic sera are not capable of simple interpretation. A number of distinct antigens in the crystalline protein was reported, despite the fact that it appeared to give only a single line in the diffusion test. This was confirmed by Ambrose and Easty (1954) who showed that when this diffusion line was examined under the interferometer it was found to be complex, with at least four razor-sharp interference peaks detectable in the narrow band of precipitate, each peak corresponding to a separate component.

Since its isolation by Pope and Stevens (1953*b*) this crystalline toxin-protein has been the subject of an intensive study which, while still incomplete, has provided no evidence to suggest that the crystalline toxin-protein consists only of the "specific toxin" as defined earlier in this paper. In the absence of any material which may be regarded as pure diphtheria toxin it is important to consider the properties of the materials available for a study of the toxin-antitoxin reaction. The earlier preparations with a value of 2170 Lf/mg. N were judged to be 90–95 per cent pure from the fact that nearly all their protein was specifically precipitable by antitoxic sera. The presence of a large number of antigens in these "toxins" as revealed by the diffusion test raised the question of what happened to them in the flocculation reaction. Pope *et al.* (1951) found that antitoxin recovered from

the toxin-antitoxin floccules by the method of Pope and Healey (1939) was contaminated by the antibodies to these antigens. The use of ^{35}S-methionine-labelled toxin by Pappenheimer and Yoneda (1957) for the reinvestigation of the toxin-antitoxin flocculation reaction is of considerable interest, but it is still of the greatest importance to know if the specific diphtheria toxin is the only protein labelled with ^{35}S-methionine, or whether other non-toxin proteins are also labelled in this way. The crude culture filtrate produced by the method of Yoneda (1957) would contain only the proteins synthesized by the *C. diphtheriae*, since it is prepared from a protein-free medium. However, the purity of preparation 2 reported by Pappenheimer and Yoneda (1957) as " almost 2400 Lf/mg. N " is much less than the purity of crystalline toxin-protein. Unfortunately no diffusion tests on these " labelled toxins " are reported, but it is improbable that they would be less contaminated with other antigens than those of similar purity studied by Pope *et al.* (1951). It would also be of considerable interest to know whether the ^{35}S-methionine-labelled toxin-antitoxin floccules yield, on pepsin treatment, antitoxin contaminated by other antibodies as found by Pope *et al.* (1951).

Diphtheria Antitoxin

The term diphtheria antitoxin should, strictly, be applied only to the antibody having the property of neutralizing the " specific toxin " as previously defined. Generally, the term " antitoxin " is applied to material obtained after the immunization of suitable animals with diphtheria culture filtrate (containing toxin or toxoid). Immunization with crude preparations containing antigens other than the specific toxin would be expected to lead to the production of other antibodies with their own specific properties. Pope *et al.* (1951) examined antitoxic sera of English, Continental and American origin by the gel-diffusion method, and demonstrated the presence of a number of antigen-antibody systems. They observed that antitoxic sera of American origin showed less contamination with these other systems and suggested that the difference could be accounted for by differences in methods of immunization, particularly by the length of the immunization course used. Bowen (1952) confirmed most of these observations and showed that 6 " purified toxins " and 17 antitoxins tested showed multiple diffusion lines. He was also able to correlate the number of lines given by an antitoxic serum with the length of the immunization course.

The purest diphtheria antitoxin isolated is that obtained by Pope and Stevens (1953*a*, *b*) ; it had a value of 211,000 antitoxic units per g. protein and was obtained from a peptic-antitoxin with an initial purity of 16,000 u./g. protein by repeated removal, by absorption, of the non-antitoxic antibodies. Despite its high purity this preparation did not behave in a uniform way with various toxin fractions isolated from crude culture filtrates. Three toxin fractions examined gave well defined and separated double zones of flocculation with this antitoxic material (Pope and Stevens, 1953*b*) and they concluded that this product was not homogeneous in an immunological sense. Thus, for antitoxin, as for toxin, pure material is not available with which to study the specific toxin-antitoxin reaction. The interpretation of results obtained from the use of impure materials needs very great care, particularly where the results are used to deduce the purity of the specific toxin or antitoxin.

Pappenheimer and Yoneda (1957) stress the need to use a *suitably chosen* horse antitoxin and state that Pappenheimer and Robinson (1937) selected for detailed

analysis antitoxins whose quantitative behaviour with toxin approximated as closely as possible to what they regarded as the " ideal system ". In their study of the reaction between ^{35}S-methionine-labelled toxin and antitoxin they used antitoxin 5353 AD which was supplied by the Biologic Laboratories, Massachusetts Department of Health. In 1952, a 40-ml. sample of this antitoxin, reputed to contain 480 flocculating units per ml., was obtained from the Biologic Laboratories, Massachusetts, through Dr. Pappenheimer and examined in these laboratories. Its value, as checked with the international flocculation standard, was found to be 383 u./ml., a difference of 97 u./ml., or about 25 per cent. On retesting this material the Biologic Laboratories obtained a value of 472 u./ml. The discrepancy in the value found for this antitoxin between the two laboratories was puzzling because at this time there had been an exchange of samples of diphtheria antitoxic sera and for these the difference in the flocculation values was less than 5 per cent. Antitoxin 5353 AD was tested for its *in vivo* value by Dr. C. L. Oakley at these laboratories : he found the following values :

At Lr/100 injected, 395 u./ml.
At Lr/500 injected, 330 u./ml.
At Lr/2000 injected, 280 u./ml.

This antitoxin has the properties of non-avid antitoxins since the toxin and antitoxin dissociate on dilution and so affect the apparent antitoxin value. Further enquiry through the Biologic Laboratories, Massachusetts, revealed that the antitoxin was produced at another laboratory ; it was a pooled collection of serum from several horses, all immunized for less than 3 months before they were bled out. It is difficult to estimate the true specific antitoxin content of sera of this kind ; clearly there was a large difference in the *in vitro* value determined in two laboratories and a variable *in vivo* value depending on the dilution at which the test was done.

With one exception all the antitoxins studied by Pope and Stevens (1953*a*, *b*) had a normal " Lr/Lf ratio " very close to 1·0 which made it possible to determine, in terms of international standard units, the amount of specific antitoxin available for combination with toxin in the flocculation reaction.

The difficulties become obvious if the two *in vitro* values for antitoxin 5353 AD are considered ; clearly they cannot both be correct. There is, therefore, doubt about the actual amount of specific antitoxin available for the reaction with the toxin in the experiments reported by Pappenheimer and Yoneda (1957). Substitution of the *in vivo* value of the antitoxin would not in any way help since this value varies with the dilution at which the tests are done.

The Composition of the Floccule Precipitate

It is of fundamental importance in the claims of Pappenheimer and Robinson (1937) and Pappenheimer and Yoneda (1957) that the floccule precipitate consists of specific " toxin " and specific " antitoxin " only and is free from any other diphtheria antigen-antibody systems regardless of whether the total nitrogen or the ^{35}S-methionine content of the precipitate is measured. Pope *et al* (1951) studied the antibody content of the product obtained by the application of the method of Pope and Healey (1939) to the floccule precipitates. They found by the gel-diffusion test that many antibodies were present. When a " purified toxin preparation " with a value of the order of 2170 Lf/mg. N was used to make the

floccules, the material recovered after peptic treatment showed the presence of 12 antibodies in the gel-diffusion test.

Pappenheimer and Yoneda (1957) did not apply this method of analysis to the ^{35}S-methionine-labelled floccule precipitates. They have found that when *C. diphtheriae* was grown in a synthetic medium containing ^{35}S-methionine and an excess of iron to prevent toxin production, the P-proteins so obtained were not precipitated in the reaction between unlabelled " toxin " and antitoxin 5353AD although they were added to the initial mixtures. This observation differs from that of Pope *et al.* (1951) who showed the presence of antibodies to the P-proteins in the floccule precipitates. Pope and Stevens (1953*a*) examined the properties of the antigens obtained by growing *C. diphtheriae* in tryptic digest medium in the presence of a large amount of iron in order to inhibit the production of the specific toxin. They concluded that in the presence of excess iron, in addition to inhibition of the specific toxin production, there was also an inhibition of the production of other non-toxic antigens normally present in crude toxin culture filtrates. The complete absorption of diphtheria antitoxic serum with these P-proteins did not eliminate all diffusion lines other than the major one: nor did it prevent the inclusion of these other non-antitoxic antibodies in the floccule precipitate.

Pappenheimer and Yoneda (1957) state that the slope of the linear portion of their curve is almost the same as the specific activity of the toxin employed when highly purified toxin is used as the antigen, and that this fact indicates that all of the toxin and antitoxin are precipitated, the increased nitrogen being solely due to toxin in this linear region. They consider that while nitrogen may be determined accurately (0·5 per cent or better) the flocculation unit is related to standard antitoxin and doubt whether Lf values are significant to 5–10 per cent. On these grounds they say that it is important to recognize that *the number of Lf/mg. N is a measure of specific activity and not of purity* (the italics are theirs). It is, therefore, interesting that Pope and Stevens (1953*c*), Norlin (1955), Katsura (private communication) and Raynaud and Relyveld (private communication) all working independently, with different toxin preparations, and with different " test antitoxins " for use in the flocculation tests, should have isolated toxin preparations (in three cases in crystalline form) with purities of 3000–3200 Lf/mg. N. It would appear from this that it is possible to determine the Lf values accurately even though different antitoxin standards are used in various laboratories. The theoretical purity predicted by Pappenheimer and Robinson (1937) from their immunochemical studies was 0·00046 mg. N per Lf or 2170 Lf/mg. N.

After a very thorough absorption of diphtheria antitoxic serum by diphtheria antigens, which were substantially free of specific toxin, Pope and Stevens (1953*a*, *b*) obtained antitoxic preparations which produced with toxins very little floccule precipitate and showed no broad equivalence zone. Commenting on these preparations Pappenheimer and Yoneda (1957) suggest that the bivalent antitoxin had been removed during the preparation and that mostly incomplete or " univalent " antitoxin remained. This is not in agreement with the results of Pope and Stevens (1953*b*) who stated that the loss of specific antitoxin, consequent on the presence of traces of true diphtheria toxin in the antigens they used for absorption, was not more than 20 per cent. Pappenheimer and Yoneda's suggestion would imply the presence in the sera of well immunized horses of about 80 per cent incomplete or " univalent " antitoxin.

Of the proteins synthesized by toxigenic strains of *C. diphtheriae* only one has well established biological properties, lethal or dermonecrotic in susceptible animals, which allows of its detection or estimation. Pope *et al.* (1951) reported the presence in crude culture filtrates of a series of proteolytic enzymes whose action was inhibited by antibodies present in commercial antitoxin preparations. These enzymes do not appear to be present in the crystalline toxin-protein, except possibly in very small amounts. However, further examination of the crystalline toxin-protein has resulted in some new observations which are given here.

EXPERIMENTAL

Crystalline Toxin-protein

Deoxyribonuclease

It was found that highly polymerized deoxyribonucleic acid isolated from salmon roe was rapidly liquefied by the crystalline toxin-protein. This property was common to a number of batches examined and it suggested the presence of a deoxyribonuclease or depolymerase. When examined by the alcohol precipitation method used for another bacterial deoxyribonuclease (McCarty, 1949) it was found that an end point was obtained with about 30 Lf units of crystalline toxin-protein. This is equivalent to about 10 μg. N or 65 μg. of toxin-protein, capable of digesting 500 μg. of deoxyribonucleic acid in 30 min. at 37°.

Crystalline toxin-protein to which one equivalent of peptic-antitoxin was added was allowed to flocculate and then centrifuged. No deoxyribonuclease activity could be detected in either the supernatant or washed floccules, re-suspended to original volume.

Until recently no reliable method has been known for the recovery of the antigens from neutral diphtheria toxin-antitoxin floccules, although the antibodies could be recovered by pepsin treatment. It has been found that by treatment with anhydrous sodium sulphite 0·3–0·5 per cent w/v at 20° the floccules first dissolve and later the antibodies are destroyed with release of the antigens. In this manner up to 90 per cent of the original specific toxin (*in vivo* tests) may be obtained from neutral floccules. Using this method it was found that deoxyribonuclease activity could be recovered from the washed floccules and none from the supernatant. The further study of this enzyme is in progress.

It is not implied that this deoxyribonuclease activity is responsible for the lethal or dermonecrotic properties of diphtheria toxin : no evidence supporting such a view has been found.

Reaction with non-flocculating antitoxin

Non-flocculating horse antitoxin was prepared by removal of non-antitoxic antibodies as described by Pope and Stevens (1953c). With the crystalline toxin-protein it gave neither flocculation nor a line in the gel-diffusion test. This antitoxin was added to crystallized toxin-protein (1000 Lf/ml.) in sufficient quantity to render it completely non-toxic when injected intradermally into guinea-pigs. Diffusion tests as described by Pope *et al.* (1951) were set up using crystalline toxin-protein as the control, and the neutral mixture prepared from it, both at equivalent original toxin concentrations, against peptic-antitoxin. Both the control toxin and the neutral mixture gave identical single lines in the same

time and of the same density. Thus, neutralization of the specific toxin with non-flocculating antitoxin did not affect diffusion speed or the ability to react typically with peptic-antitoxin in producing a diffusion line. This result suggests that neutralization of the specific toxin by this antitoxin leaves other antigens unaffected and that these are therefore free to diffuse and react with their antibodies present in the peptic antitoxin. Since the density of the diffusion line was unaffected it may also mean that the specific toxin forms only a small part of the crystalline toxin-protein.

Digestion with trypsin

The action of crude trypsin, prepared by the method of Cole and Onslow (1916) from ox pancreas, and of crystalline trypsin, on crystalline diphtheria toxin-protein has been examined. Both produce similar results; the following work was carried out with the crude enzyme.

Toxin solution containing 1000 Lf/ml. was used at pH 8·0 and crude trypsin solution (2 ml. per 100 ml. toxin) added. Digestion was allowed to proceed at 20° and samples were removed at various times. To these, soya-bean trypsin inhibitor was added to stop further enzyme action. The samples were tested for *in vitro* and *in vivo* values, and examined for diffusion lines. The results are shown in the Table. All samples up to 8 hr. digestion showed only a single flocculation value. From 12 to 48 hr. all showed clearly separated double zone flocculations. Examination showed that all Lf values up to 8 hr. and the lower values from 12 to 48 hr. fell on a smooth curve. The high Lf values found from 12 to 48 hr. were of the same order, indicating that this antigen had been little affected by digestion with trypsin. Until digestion has proceeded for about 8 hr. or more it is not possible to detect the two zones of flocculation because they are not at that time well separated. This observation has been repeated many times on different preparations of crystalline toxin.

The *in vivo* tests show the rapidity with which the specific toxicity of the crystalline toxin is destroyed by trypsin. It will be seen from the Table that the number of Lr doses per ml., expressed as a percentage of the original, falls more rapidly than the Lf value. The loss of specific toxin as shown by the reduction in the number of Lr doses per ml. appears to be due to destruction and not to any toxoiding process.

The change in the diffusion lines is also shown in the Table. The control crystalline toxin-protein shows only the single diffusion line but after 2 hr. tryptic digestion three separated lines were obtained.

After 6 hr. digestion four lines were found and after 26 hr. two lines remained. During the digestion by trypsin the trichloracetic acid-precipitable proteins are rapidly digested except for a portion of the protein which is very resistant to trypsin. It is this resistant protein which is responsible for the high Lf value found between 12 and 48 hr. and the single diffusion line left at 48 hr. From other (unpublished) work there is evidence to suggest that more than one protein (and antigen) is rapidly digested by trypsin.

These observations on the action of trypsin on crystalline toxin-protein provide further support for the view of Pope and Stevens (1953*a*) and Pope (1954*b*) that the "flocculation envelope" contains several antigens and their antibodies. It is impossible at present to say which of the antigens present in crystalline toxin-protein is responsible for the specific lethal and dermonecrotic effects.

TABLE.—*The Action of Trypsin on Crystalline Diphtheria Toxin-protein*

Hour's digestion at 20°	Per cent of original values (a) *In vitro*	Per cent of original values (b) *In vivo*	Diffusion lines (c)
0	100	100	1
2	75·5	55	3
4	54·0	33	3
6	37·5	22	4
8	27·0	17	3
12	20·0 and 73·0	8	3
20	16·5 „ 67·0	4·5	2
26	7·5 „ 71·0	<1·0	2
48	5·0 „ 70·0	<0·5	1

Initial values for toxin 1000 Lf/ml. and 940 Lr doses/ml. :

(*a*) Up to 8 hr. digestion, only one zone of flocculation : at 12–48 hr. there were two zones of flocculation.

(*b*) Intracutaneous tests in guinea-pigs against standard antitoxin.

(*c*) Number of diffusion lines on Ouchterlony (1948) agar plates. The one line at 48 hr. is due to the trypsin-resistant protein and corresponds with the antigen responsible for the high Lf value.

A small amount of antitoxin 5353 AD was available with which to examine the behaviour of the crystalline toxin-protein after 12 hr. digestion. It was found that this antitoxin gave the two zones of flocculation obtained with peptic-antitoxin, and in addition it showed three diffusion lines in the gel-diffusion test. It is quite clear, therefore, that antitoxin 5353 AD does contain antibodies to other antigens shown to be present in the crystalline toxin-protein complex and there is no reason to expect that they would behave in a manner different from the similar antibodies present in the peptic-antitoxin used in this work.

In addition to the experimental results given above, a number of other methods all of which produce separation of the antigens in the crystalline toxin-protein, are under investigation (Pope and Stevens, unpublished).

The properties of the crystalline toxin-protein recall the remark of Fraenkel-Conrat (1956) : " Crystalline and homogeneous proteins of a few years ago are now often fruitful sources for the isolation of new pure proteins ".

DISCUSSION

The purest form in which diphtheria toxin has yet been isolated is the crystalline toxin-protein. It has been shown to be a complex of several different antigens on the grounds that it contains an enzyme with the properties of a deoxyribonuclease ; that after neutralizing the specific toxin with non-flocculating antitoxin the diffusion line against peptic-antitoxin is unaffected and that on digestion with trypsin, only part of the protein is digested, double-zone flocculation is produced and the single line in the diffusion test is, at first, replaced by a three-line system. Trypsin destroys the specific toxicity at a faster rate than the labile flocculating system, yet still leaves a resistant protein which flocculates at about 70 per cent of the original Lf value.

The deoxyribonuclease has been recovered from the toxin-antitoxin floccules produced from this crystalline " toxin " after destruction of its antibody. It therefore formed part of the floccule complex. Similarly, the antigens disclosed

by the action of trypsin form part of the floccule complex even when the crystalline "toxin" is used to produce the floccules. At present it is impossible to determine what proportion of the crystalline protein complex is accounted for by the specific toxin. The presence of three to four distinct antigens, each with a specific antibody, has been shown in this paper but additional work (Pope and Stevens, unpublished) has made it clear that at least five such antigens are present.

Until further knowledge is obtained about the properties of these antigens it is unjustifiable to consider that they all have the properties of the specific toxin. In fact, the enzyme deoxyribonuclease appears to have no lethal or dermonecrotic properties, and the experiments with trypsin suggest that two antigens, responsible for the two zones of flocculation, are not responsible for the specific toxicity.

With these new observations to be considered it is difficult to avoid the view that the floccule precipitate contains antigen-antibody systems unrelated to the essential specific toxin-antitoxin complex. Whether measured in terms of nitrogen or ^{35}S-methionine-labelled protein present in the floccules, the contribution made by these other systems must be taken into account, and simple deduction about the purity of toxin from immunochemical studies should be regarded with caution.

SUMMARY

The properties of diphtheria toxin and antitoxin have been considered in relation to the flocculation reaction.

Crystalline diphtheria toxin-protein has been shown to contain deoxyribonuclease which is precipitated with the floccule precipitate. The enzyme has been recovered from the floccules.

A method for the liberation and recovery of the antigens present in neutral floccules is given. Both the specific diphtheria toxin and the deoxyribonuclease were recovered after destruction of their antibodies.

It has been shown that after complete neutralization of crystalline toxin-protein by non-flocculating antitoxin, the diffusion line against peptic-antitoxin is unaffected.

The action of trypsin on crystalline toxin-protein has been studied, and results showing the inhomogeneity of this toxin preparation are given.

I should like to thank Dr. A. M. Pappenheimer for arranging a supply of antitoxin 5353 AD; also Dr. J. A. McComb (Biologic Laboratories, Boston, Massachusetts) and Dr. H. D. Piersma (Lederle Laboratories) for much help and information regarding this antitoxin. I am also indebted to Professor C. L. Oakley for *in vivo* tests, to Dr. R. O. Thomson for a supply of deoxyribonucleic acid and especially to my colleague Miss M. F. Stevens for help throughout this work. My thanks are also due to Miss M. Barr who drew my attention to the fact that when using toxoid, treated with 0·3 per cent metabisulphite to remove excess formaldehyde, to produce floccules, these at first formed normally but later dissolved again.

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VACCINATION DURING THE INCUBATION PERIOD

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FOR almost 60 years the attention of epidemiologists and immunologists has been drawn to the problem of vaccination during the incubation period, and the so-called " negative phase " after vaccination.

Since the work of Wright (1901) evidence may be found in the literature suggesting that vaccination during the incubation period caused a worse course of the disease and an increased mortality. Nevertheless, opposite views may also be found. The problem has very important practical implications. It is known, for instance, that pertussis vaccine is sometimes given to children who have just come into contact with the disease, *i.e.*, who are already in the incubation period or may be infected very soon after the administration of the vaccine. This, of course, should not be confused with the therapeutic use of pertussis vaccine in late stages of the disease.

Because we have seen more severe pertussis after vaccination of children, who were at the time in contact with the infection, we decided to study this question using mice as an experimental model.

MATERIALS AND METHODS

Bacterial cultures

Haemophilus pertussis strain 637, freshly isolated and adapted for intranasal infection in mice was used. The strain is stored in lyophilized form and its virulence is constantly controlled. For infection a 72-hr. culture on Bordet-Gengou medium from the first subculture of the lyophilized strain was used. A suspension was prepared in saline, containing 10^{10} organisms per ml. (based on spectrophotometric control of density). The suspension was titrated in mice by intranasal inoculation of 0·05 ml. volumes under light ether anaesthesia. Only mice which aspirated the infective dose well were taken in the experiment. Mice which sneezed or swallowed were excluded. The animals were observed for 21 days. Animals which died were dissected, and blood from the heart and lungs was tested for the presence of *H. pertussis*. The LD_{50} was found to be 10^8–10^9 organisms per 0·05 ml. dose.

Animals

Albino mice of the H strain in the weight range 11–13 g. were used. They were fed with a standard diet and kept under constant conditions.

Pertussis vaccine

" Preperba " vaccine, containing 3×10^{10} organisms per ml. was used. For experiments, the vaccine was diluted by saline 1/4 and 0·5 ml. was given intraperitoneally. This dose is completely innocuous for control mice. In experiments carried out in 1954 we infected mice and gave the vaccine on the 1st, 2nd and 3rd day after infection and in a second experiment they were infected on the 1st, 2nd, 3rd and 4th day after vaccination. The first experiment was repeated twice, the second four times. Groups of 10 mice were used for each procedure.

In later experiments groups of mice, which had received varying infective doses, were vaccinated 5, 24, 48 and 72 hr. after infection.

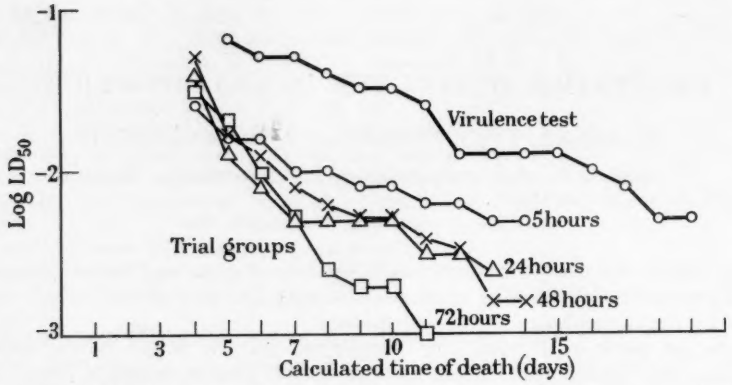

FIGURE.—Joined points represent the values of lethal doses calculated by the method of Kärber from the numbers of surviving animals (given also in Table III but for selected days only), for separate days of duration of the whole assay.

RESULTS

The results of the first group of experiments may be seen in Tables I and II. The results in Table I show clearly that vaccination after infection, during the incubation period, increases the death rate significantly.

Table II shows the same increase of the death rate in animals infected in the first days after the vaccination (negative phase). Qualitative analysis of the data

TABLE I.—*Death Rate of Mice Vaccinated* 1–3 *Days after Infection*

Interval between the infection and treatment	Assay No. XII	Assay No. XV
1 . .	10/10*	10/10
2 . .	6/10	8/10
3 . .	6/10	9/10
Control test . .	5/10	5/10

* Number dying/number inoculated.

TABLE II.—*Death Rate of Mice Infected* 1–4 *Days after Vaccination*

Interval between the treatment and infection	Assay No. X	Assay No. XVI	Assay No. XI	Assay No. XVII
1 . .	10/10	9/10	5/10	6/10
2 . .	10/10	8/10	5/10	5/10
3 . .	10/10	8/10	3/10	6/10
4 . .	7/10	6/10	—	—
Control test . .	5/10	5/10	3/10	3/10

clearly shows the difference between the trial and control groups. With 20 degrees of freedom the sum of χ^2 is 31·3 ($P = < 0{\cdot}05$).

Table III gives the results of our later experiments and confirms our earlier results.

TABLE III.—*Number of Mice Surviving when Vaccinated at Varying Times after Infection*

Interval between infection and treatment (hours)	Challenge dose	Number of mice surviving challenge on day: 1	3	5	7	10	15
5	10^9	10	4	2	1	1	0
	10^8	10	9	5	4	3	0
	10^7	10	10	10	10	10	9
24	10^9	10	8	4	1	1	0
	10^8	10	10	3	3	3	1
	10^7	10	10	9	8	8	7
48	10^9	10	9	1	0	0	0
	10^8	10	10	6	4	2	1
	10^7	10	10	10	10	10	9
72	10^9	10	9	4	2	0	0
	10^8	10	8	5	3	3	0
	10^7	10	10	9	7	5	3
Virulence test	10^9	10	7	4	4	4	3
	10^8	10	10	9	8	6	4
	10^7	10	10	10	10	10	9

Especially interesting is the finding that the lethal doses decrease as the interval between challenge and injection of the vaccine is prolonged. The assays were arranged in order to treat the groups of challenged mice with vaccine after 5, 24, 48 and 72 hr. respectively, and after 0 hr. which properly speaking is the virulence test of the strain of *H. pertussis* used in the assays. The period of duration of these assays is very long (15 days) ; we did not select a certain day (for example the 10th), reckon the dead animals and calculate the LD_{50}, and then compare the results. We preferred rather to calculate the LD_{50} for all separate days for the duration of the assays and not only to take into account the differences between the LD_{50} but also the death rate of the animals. If the lines of the trial groups are quite different from the control one, the meaning of the result is the same as in preceding assays, *i.e.*, qualitative.

DISCUSSION

Our results from intranasal infection with *H. pertussis* in mice give clear evidence that vaccination in the first days after the infection significantly raises the death rate of the infected animals. This fact was found repeatedly over a long period and the great number of mice used in these experiments, 1200, further excludes the possibility of chance results. We also found evidence for the existence of a negative phase, *i.e.*, an increase in death rate of animals vaccinated 1–4 days before challenge.

There seems to be a discrepancy between our results concerning the negative phase and the work of Evans and Perkins (1955). They found that an intraperitoneal injection of pertussis vaccine has some protective influence on challenge even after 5 hr. and especially after 1 or more days. However, they infected the animals by the intracerebral route. We are therefore repeating our experiments to compare them directly with the intracerebral challenge of Evans and Perkins,

but this work is not yet finished. From other experiments, made by Rašková in collaboration with us, it is known that a second contact with an antigen in a short interval causes deep physiological disturbances of a non-specific character (Rašková *et al.*, 1952, 1953, 1955, 1956). These observations are studies of the pharmacological properties of various toxins (*Shigella shigae*, endotoxin of *Salmonella typhi* and streptolysin O). In animals treated with small doses of the toxins 2–21 days previously, they found intensified reactions in comparison with animals which were for the first time in contact with the toxin. Changes of conditioned reflexes of an inhibitory character in rats were more severe. The administration of toxins in humorally isolated chemoreceptor areas caused severe circulatory disturbances. The conductivity of motor nerves was more impaired than in control animals. However, when the toxins previous to the corresponding experiments were applied several times, an adaptation of a non-specific character occurred. Conditioned reflexes and nerve conductivity were no more impaired by the application of the same as well as by the administration of other, antigenically non-related toxins.

It is evident that no direct application of the results of experiments in mice to humans is possible. We think, however, that these experiments suggest an unfavourable influence of vaccination during the incubation period.

SUMMARY

It has been shown that vaccination of mice with pertussis vaccine in the first few days after intranasal infection with *Haemophilus pertussis* significantly raises the death rate of the animals. The significance of the observation is discussed.

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A HISTOCHEMICAL STUDY OF THE ESTERASES OF RAT THYROID AND THEIR BEHAVIOUR UNDER EXPERIMENTAL CONDITIONS

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It has been known for a long time that changes in thyroid activity are accompanied by definite cytological changes in the gland, but little is known about the associated enzyme changes or their significance. A proteolytic enzyme has been found by de Robertis (1949) in the colloid of active thyroid follicles, and Schachner, Franklin and Chaikoff (1943) obtained evidence that the cytochrome–cytochrome oxidase system was involved in secretory activity. Okkels (1934) has related the distribution of oxidase granules in the thyroid of the guinea-pig injected with thyrotrophic hormone to the functional state of the gland and peroxidase activity was demonstrated in the cells of the thyroid follicles by Dempsey (1944), Glock (1944), and de Robertis and Grasso (1946).

Alkaline glycerophosphatase was demonstrated in the capillaries of the mammalian thyroid by Gomori (1941) and by Kabat and Furth (1941). Wolf, Kabat and Newman (1943) mention the presence of acid phosphatase in the nuclei and cytoplasm of the thyroid epithelium but give no details as to its localization in the cell. Dempsey and Singer (1946) found alkaline glycerophosphatase in the follicular epithelium of peripheral inactive follicles in the rat thyroid and acid glycerophosphatase in the cells of the central active follicles. They also demonstrated glucose phosphatase and fructose diphosphatase in the capillary endothelium.

With regard to the carboxylic acid esterases, Gomori (1946), using Tween 40 or 60 and Product 81 as substrates, found no lipase in the thyroid of the rat or of any of the other species which he examined. Huggins and Moulton (1948) quantitatively demonstrated the presence of a non-specific esterase in the rat thyroid with *p*-nitrophenyl propionate as substrate and Weber (1954), using α-naphthyl acetate and the Tweens, studied the enzymes of rat thyroid after treatment with thyroxine and thyrotrophic hormone. He found an enzyme activated by sodium taurocholate and not inhibited by 0·001 M eserine and suggested that it was an aliesterase, probably a lipase. Dejardin (1955) described the distribution of pseudocholinesterase in guinea-pig thyroid. He found this enzyme to be present in so-called replacement or chromophile cells.

The present report deals with the nature and distribution of rat thyroid esterases using *o*-acetyl-5-bromoindoxyl (5. Br. I) and acetylthiocholine (AThCh) in association with various inhibitors. The two enzymes which were identified have been studied under various experimental conditions.

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MATERIAL AND METHODS

Rats.—Male albino rats weighing approximately 200 g. were used.

Tissues.—The rats were killed with coal gas and a block of tissue containing the thyroid attached to the trachea was immediately removed and fixed in cold (4°) 15 per cent formalin containing 1 per cent $CaCl_2$ (pH 7·4) for 24 hr. Sections were cut at 15 μ on a freezing microtome, washed in distilled water for a few minutes and then incubated in the substrate mixtures. In order to counteract the error arising from variations in thickness, 8–10 sections from each gland were taken in almost unbroken succession and placed in the individual incubation mixtures.

Substrates.—1. *o*-Acetyl-5-bromoindoxyl. This was used according to the method described by Pearse (1953) except that the pH used was 6·2.

2. Acetylthiocholine. Gomori's (1952) modification of the Koelle method was used.

Incubation (37°).—With the indoxyl substrate the sections were incubated for 45 min. With acetylthiocholine as substrate the optimum time was found to be 8 hr.

Inhibitors.—The following inhibitors were used for the identification of the enzymes: 62C47 (1 : 5-bis-(4-trimethylammonium-phenyl)pentan-3-one diiodide, 0·001 M; *iso*-OMPA (tetra-*iso*propyl pyrophosphoramide), 0·001 M; E600 (diethyl-*p*-nitrophenyl phosphate 0·0001 M; silver nitrate, 0·02 M; sodium taurocholate, 0·05 M; sodium dodecyl sulphate, 0·01 M; hydrocinnamic acid, 0·01 M; hydroxylamine, 0·01 M; sodium fluoride, 0·01 M.

When the inhibitors were used the sections were first incubated in solutions of these for 1 hr. and then transferred to the substrate mixture containing the same concentration of inhibitor, except in the case of the irreversible inhibitors.

Fat stains.—In order to correlate enzyme changes with fat metabolism the following stains were employed:

For neutral fats: Oil Red O, Sudan III and IV and Phosphine 3R. For phospholipids: Sudan Black B and Baker's acid haematein method.

Experiments

For this purpose a total of 26 rats was used, divided into the following groups:

Group I.—Daily subcutaneous injections of 1 U.S.P. unit thyroid stimulating hormone (T.S.H.) were given to 3 rats. The hormone preparation used was Armour's Thytropar freshly dissolved in saline before use. These rats were killed on the 2nd, 4th and 8th days after starting the injections.

Group II.—A saturated solution of 4-methyl-2-thiouracil in place of the ordinary drinking water was given to 10 rats, of which one was killed on alternate days from the 4th to the 22nd day inclusive.

Group III.—Daily injections of 3 μg./100 g. rat of L-thyroxine subcutaneously were given to 10 rats in drinking the saturated solution of 4-methyl-2-thiouracil. The thyroxine was made up daily as follows: 1 tablet of sodium L-thyroxine (0·1 mg.) was dissolved in 50 ml. of normal saline to which had been added 40 mg. sodium carbonate. These rats were killed at the same times as those of group II.

Group IV.—Auto-implants of half of the thyroid gland were done on a group of 3 rats according to the method described by Dempster and Doniach (1955). These were killed on the 9th day after the operation. Control animals were killed with all the experimental animals.

RESULTS

Histochemistry of the Thyroid Esterases

Indoxyl esterase.—The enzyme demonstrated by using *o*-acetyl-5-bromoindoxyl is present in all the acini throughout the gland, but in slightly higher concentration in the peripheral and isthmic acini than in those in the centre of the thyroid lobes. It is present throughout the cytoplasm of the individual cells, but usually shows a higher concentration near the luminal border of the cell (Fig. 1). In some acini this appearance is so distinct that the precipitate of 5-5′-dibromoindigo forms an almost complete circle between the nuclei and the lining

of the follicle. No enzyme could be detected in the nuclei and in the normal gland the colloid was completely devoid of enzyme activity.

The reaction of this enzyme towards the various inhibitors and activators is given in the Table.

TABLE.—*Effect of Inhibitors and Activators on Thyroid Indoxyl Esterase*

Inhibitor	Per cent activation	Per cent inhibition
Sodium fluoride 0·01 M	—	—
Hydroxylamine 0·01 M	—	—
$AgNO_3$ 0·02 M	—	100
Cysteine 0·01 M	30	—
Hydrocinnamic acid 0·01 M	—	—
Sodium dodecyl sulphate 0·01 M	—	30
E600 0·0001 M	—	100
Sodium taurocholate 0·05–0·01 M	30	—
62C47 0·001 M	—	—
iso-OMPA 0·001 M	—	—

Because both 62C47 and *iso*-OMPA had no effect on the indoxyl esterase it cannot be a cholinesterase, either specific or non-specific. Inhibition by E600 confirms its general identity as a carboxylic acid esterase and inhibition by sodium dodecyl sulphate suggests that it may be a lipase. Cysteine activation and silver nitrate inhibition characterize those esterases which probably act *in vivo* as peptidases, and stimulation by sodium taurocholate, though often considered to favour lipase rather than simple esterase, we have found to characterize peptidase-type esterases elsewhere in the body (Pepler and Pearse, 1956).

Cholinesterase.—The enzyme in the thyroid parenchyma was completely inhibited by 0·001 M *iso*-OMPA and not at all affected by 62C47 at the same concentration. This indicates that it is a non-specific or pseudocholinesterase. Acetylcholinesterase was limited to the plexuses of fine cholinergic fibres surrounding the follicles.

The pseudocholinesterase always had a patchy distribution in that the centrally placed follicles regularly contained small amounts of the enzyme whereas peripherally the majority of follicles showed no enzyme activity. However, occasional follicles at the periphery of the lobes and in the isthmic portion, although lined by flat cuboidal epithelium, contained a large amount of the enzyme (Fig. 2). As a rule the enzyme was evenly distributed in the cells of individual follicles, but occasionally only some of the cells showed enzyme activity. Frequently the enzyme was localized in circular bands or dispersed in small groups of cells without any regularity. In a given cell the enzyme appeared to be concentrated in two sites, around the nucleus and near the luminal border. In the occasional follicle with a very intense enzyme reaction in the cells, slight staining was also observed in the colloid, but otherwise no enzyme could be demonstrated here. The nuclei were always free of enzyme activity.

Experimental Results

Group I (TSH).—In the rat killed on the 2nd day of the experiment the amounts of both the indoxyl and pseudocholinesterase appeared to be within normal limits. However, the rats sacrificed on the 4th and 8th days showed a definite decrease of the indoxyl esterase (Fig. 3). The pseudocholinesterase

showed a decrease per cell and per lobule as a whole, but in areas with strong enzyme activity the reaction still remained very intense.

Group II (*Thiouracil*).—The rat killed 4 days after the beginning of the experiment showed a distinct decrease of the indoxyl esterase in the central reactive part of the lobe. In addition there also appeared to be a redistribution of the enzyme in the cells so that, although it was still present throughout the individual cells, there was a relatively heavier concentration near the luminal border. At 8 days the decrease in enzyme activity was apparent in the whole gland. At 12 days the basal parts of the cells in some follicles were entirely free from enzyme and the luminal part of the cell showed only faint activity (Fig. 4). Some of the follicles, especially in the central part of the lobe, were almost devoid of enzyme activity. In the rats killed at later stages the overall picture remained stationary although the cells were even more hypertrophic.

Owing to the patchy distribution of the pseudocholinesterase, it was difficult to assess early changes in the amounts of the enzyme with any accuracy. In the rats killed on the 8th day however, it became clear that there was a decrease in this enzyme. In the later stages of the experiment, that is, in the rats killed from the 16th–22nd day, only occasional cells were found in the 8 sections examined which showed any residual enzyme activity.

Group III (*Thyroxine–thiouracil*).—In the majority of rats the dosage of thyroxine employed seemed adequate to prevent stimulation of the thyroid cells by thyrotrophic hormone and in these the amounts of both the indoxyl and pseudocholinesterase showed no significant variation from that of the control series.

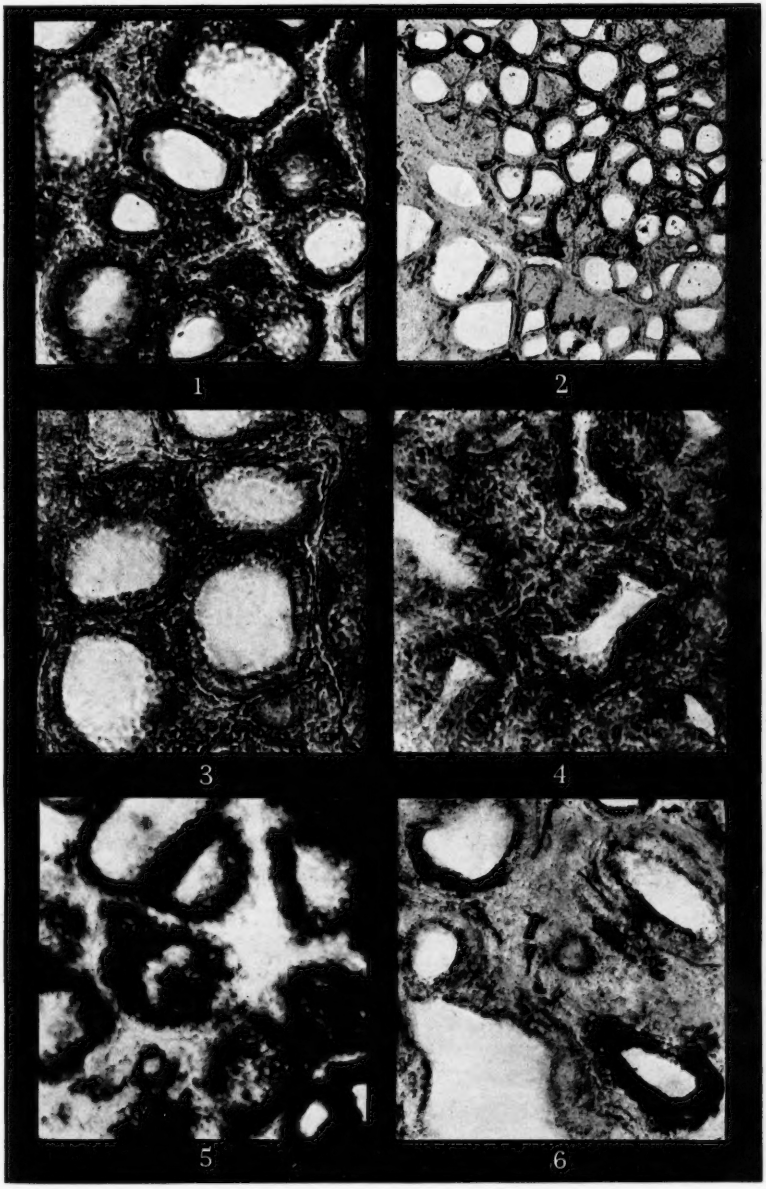
Group IV.—As shown in Fig. 5 and 6, the indoxyl and pseudocholinesterases could still be demonstrated in the auto-implants removed on the 8th day after the operation.

DISCUSSION

Although the presence of a non-specific esterase in the thyroid is fairly well established (Huggins and Moulton, 1948), there is still considerable controversy with regard to the presence or absence of a lipase (cf. Gomori, 1946; Weber, 1954). After inhibition of the pseudocholinesterase (Table I), the reaction of our indoxyl esterase towards $AgNO_3$, cysteine and E600, suggested that the possibility of its being a lipase could not be ruled out altogether, especially in view of the fact that it was stimulated by taurocholate. In order to pursue this matter further we decided to correlate variations of the enzyme under experimental conditions with the lipid metabolism of the gland. In this respect further difficulties were encountered because although Dempsey (1949) and Hassler, Gislen and Brolin (1953) were able to demonstrate lipids in the epithelial cells, the former claimed an

EXPLANATION OF PLATES

FIG. 1.—Indoxyl esterase in rat thyroid (control). × 190.
FIG. 2.—Pseudocholinesterase in rat thyroid (control). Note the patchy distribution of the enzyme in the follicles. × 50.
FIG. 3.—Indoxyl esterase in rat thyroid (T.S.H. for 8 days). × 190.
FIG. 4.—Indoxyl esterase in rat thyroid (thiouracil for 12 days). × 190.
FIG. 5.—Indoxyl esterase in auto-implant of thyroid removed after 9 days. × 205.
FIG. 6.—Pseudocholinesterase in thyroid auto-implant. × 205.



Pepler and Pearse.

increase of cytoplasmic lipids under conditions of stimulation of the thyroid, whereas the latter stated that the amount of lipid was decreased. It therefore seemed necessary to repeat this work in the present series of experiments. We were, however, unable to demonstrate any significant amount of neutral fat or phospholipid with the methods used in either the control rats or the rats stimulated with thiouracil. In an occasional section stained with Oil Red O there was a suggestion of a slight pink staining near the luminal border of the cells, but this was by no means a constant finding. We believe, therefore, that there is no correlation between the indoxyl esterase and either phospholipid or neutral fat metabolism.

Recent work (Kirschner, 1953; Koch, 1954) has indicated that the cholinesterases may be involved in the active transport of ions (Na^+ and Cl^-) through cell membranes. This led us to investigate the possibility that the pseudocholinesterase in the thyroid might have a similar function in relation to the metabolism of iodide. It is well known that both acetyl- and pseudocholinesterase are present in peripheral nerves (Cavanagh and Webster, 1955; Cavanagh, Thompson and Webster, 1954) and it was therefore necessary to make sure that the pseudocholinesterase was not associated in any way with the nerve supply of the gland. In order to prove this, auto-implants of thyroid were done and the implants were examined after sufficient time had elapsed for the nerves to degenerate. By this means it was possible to show that the enzyme belonged to the thyroid cells proper and was not associated with the nerve supply. The findings of Morton, Perlman and Chaikoff (1941) that thyrotrophic hormone increases assimilation of iodine by the thyroid gland suggested to us that the action of this hormone might be explained on the basis of an increase in enzymes concerned with the trapping of iodine. In view of the work of Kirschner (1953) and Koch (1954) it was thought that the enzyme might be a cholinesterase. The effect of T.S.H. on the pseudocholinesterase was forthwith tested but, if anything, it caused a decrease in the amount of enzyme in the gland rather than an increase. This decrease, however, might have been unassociated with any direct action of the hormone on the enzyme system and could have been due to the associated hypertrophy of the cells. In order to check these results a second group of rats was given thiouracil. The resultant hypertrophy of the thyroid in this group, produced by endogenous thyrotrophic hormone stimulation, was again associated with a definite decrease in the pseudocholinesterase content of the gland. Although it seemed unlikely that thiouracil would affect this enzyme directly, we thought it necessary to control this. Thyroxine was therefore given to a third group of rats (in addition to the thiouracil) in doses that would suppress endogenous pituitary thyrotrophic hormone production (Griesbach, Kennedy and Purves, 1949; Purves and Griesbach, 1946). The results obtained confirmed the fact that the blocking effect of thiouracil was not related to these enzyme systems.

The indoxyl esterase behaved similarly to the pseudocholinesterase under the experimental conditions studied and in this respect our results differ from those of Weber (1954) who found an increase of the α-naphthyl esterase under conditions of stimulation of the thyroid.

SUMMARY

The nature and distribution of enzymes which hydrolyse *o*-acetyl-5-bromoindoxyl and acetylthiocholine in the rat thyroid have been investigated.

On the basis of inhibition studies we suggest that the indoxyl esterase may be a peptidase. The enzyme hydrolysing acetylthiocholine is a pseudocholinesterase.

Attempts to correlate the level of pseudocholinesterase with the level of iodine metabolism were unsuccessful.

The decrease of pseudocholinesterase and indoxyl esterase (peptidase) produced by stimulation of the gland must be regarded as relative rather than absolute, due to hypertrophy of the cells.

We are grateful to Dr. I. Doniach for his help and advice with the experiments, and to the Director of the Wellcome Research Institute for the gift of the inhibitor 62C47.

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THE RESPONSE OF THE DOG TO ORAL L-HYDRAZINOPHTHALAZINE (HYDRALAZINE)

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L-HYDRAZINOPHTHALAZINE (hydralazine) was introduced by Gross, Druey, and Meier (1950) as an hypotensive drug. It was subsequently used widely in the treatment of human hypertension (Schroeder and Morrow, 1953; Kuehns, Meyer-Hofmann and Hoepfner, 1953; Essellier, Luescher and Morandi, 1953; Harris and Turner, 1954).

Following prolonged administration of large doses of hydralazine to patients with hypertension, several reports appeared of undesirable side effects. Attention was drawn to instances in which general ill-health, malaise, fever, progressive anaemia and splenomegaly were combined with arthralgia, joint swelling and weight loss (Henn, Parkin, Hargraves and Odel, 1955; Muller, Rast, Pryor and Orgain, 1955). This so-called " hydralazine syndrome ", in its early phases, was considered to resemble rheumatoid disease. The suggestion of an association with the diseases of connective tissue was confirmed later when more severe illness developed. It was found that if the drug was given to patients already showing early evidence of the hydralazine syndrome, changes sometimes developed which in some ways resembled those found in disseminated lupus erythematosus (D.L.E.). A skin rash, occasionally with a facial distribution of the butterfly type, with a suggestive histological appearance (Weiss and Swift, 1955), renal damage with albuminuria, and alterations in the plasma proteins were described. Search for " L.E. cells ", using plasma from those patients and normal human leucocytes, was successful in a few cases. The syndrome was, however, distinguished from D.L.E. by the rapid disappearance of signs and symptoms following withdrawal of the drug. Nevertheless, it was occasionally possible to demonstrate the production of L.E. cells for several months after treatment had stopped.

Recently, Comens (1956) reported that the feeding of large doses of hydralazine to dogs resulted in the production of changes some of which were similar to those occurring in the human hydralazine syndrome, and which he considered to be in many respects similar to D.L.E. Weakness, weight loss, anaemia and altered plasma proteins were associated with the presence in the plasma of a factor which induced the formation of L.E. cells. Histologically, the appearance of the kidneys was accepted as consistent with the diagnosis of D.L.E., but no lesions were found in other organs or in the skin.

Because of the lack of a satisfactory experimental replica of D.L.E., the report of Comens (1956) was clearly of considerable importance, and the purpose of the present experiment was to re-examine his claims.

MATERIALS AND METHODS

Four large young mature collie dogs (1, 2, 4 and 5) and one collie bitch (3) were used. One of the dogs served as a control for haematological investigation, while for histological purposes, the kidneys from four other untreated animals were also available. The animals

were healthy and well nourished. Their diet, which was identical with that which maintained the rest of the large dog colony in good health, comprised 1 large dog biscuit and 1 pint of fresh milk at 9 a.m., with 1 pint of a composite stew at 3 p.m. each day. The stew contained small dog biscuits, dried horse meat and fresh horse meat in varying proportions, and was boiled before use. Unlimited water was supplied. Food and water were given in clean, but often chipped, enamel bowls.

Hydralazine was used in the form of 50 mg. and 200 mg. tablets, and as 200 mg. gelatin-coated capsules.

Blood, obtained by venepuncture, was mixed with Wintrobe's anticoagulant.

Haemoglobin (Hb.) was measured colorimetrically by the alkaline haematin method of Clegg and King (1942) and was compared with the artificial standard of Gibson and Harrison (1945).

Erythrocyte sedimentation rates (E.S.R.) were measured in Westergren tubes, and the fall in mm. was read at the end of 1 hr.

The packed cell volume (P.C.V.) was measured after centrifugation at 3000 r.p.m. for 30 min. in a M.S.E. haematocrit centrifuge.

Serum proteins were measured by the method of Gornall, Bardawill and David (1949).

The electrophoretic pattern of the serum proteins was examined by means of a modified Kelab apparatus, the runs being made for 4–5 hr. at 4°, using a veronal buffer of pH 8·6 (0·01 M). The P.D. was 300 volts over a Whatman No. 3 paper of 20 cm. length.

Preparations made by the modification of the method of Carrera, Reid and Kurnick (1954), introduced by Comens (1956), were searched for L.E. cells.

Histological methods

Tissue taken for histological examination was divided and placed in two fixatives, neutral formol saline and Helly's fluid. Bone was decalcified in a formic acid-sodium citrate mixture. After fixation, material was dehydrated in alcohol, cleared in chloroform and embedded in paraffin wax. Sections were cut at 3–6 μ according to the tissue, and stained with haematoxylin and eosin, and by the periodic acid-Schiff method.

Procedure

Each animal was weighed, and blood withdrawn by venepuncture. The electrophoretic pattern of the serum proteins was examined, and preparations made for L.E. cells. The daily feeding to dogs 1, 2, 3 and 4 of 200 mg. hydralazine in the form of four 50-mg. tablets then began. The drug was given each day without exception. The tablets were administered by hand before the principal feed ; they were not concealed in the food. When the experiment had been in progress for 25 days the 50-mg. tablets were abandoned, and the drug given as single 200-mg. gelatine capsules. For 13 weeks this procedure was unchanged. For the remaining 12½ weeks of the experiment the drug was added to the main feed at the time of its preparation.

The weight of each animal was recorded weekly by an independent observer.

Blood counts and plasma protein estimations were made at 2–4 weekly intervals. The estimates were made by observers who were in no way familiar with the progress of the experiment. Preparations for L.E. cells of dog serum and human leucocytes were made personally at intervals of 2–4 weeks. Both fresh dog sera, and sera kept for varying times at −20° were examined in this way. The leucocytes were obtained from four healthy human males. From each preparation multiple films were made, stained by a mixture of Leishman and Giemsa stains, and searched personally for prolonged periods on repeated occasions. At the close of the experiment each animal was killed by the rapid intravenous injection of 10–15 ml. of nembutal. Organs for histological examination were immediately removed and placed in fixative.

RESULTS

General Behaviour

The administration of hydralazine tablets had an immediate effect ; the dogs ate with obvious distaste, occasionally leaving an entire feed untouched, or regurgitating part or the whole. Within a few days they became accustomed to the administration of the drug, and food intake returned to normal.

As time passed it became increasingly difficult to administer the drug. In an attempt to overcome this, the taste of the drug was concealed by enclosing each dose in a gelatine capsule. No immediate change in the severe systemic response of the animal to the drug was observed. Convulsions were seen only in dog 3. This animal began to have generalized tetanic spasms 22 weeks after administration of the drug began. The convulsions were often precipitated by stimuli such as feeding. Between attacks the animal remained in a state of increased consciousness and apprehension. It was nervous, and difficult to feed, responding to slight noises by prolonged barking. To a lesser extent this type of abnormal behaviour was observed in the other three animals. The extreme illness with inability to stand described by Comens (1956) was not seen.

Weight

During the first 6 weeks of the experiment, loss of weight was severe (Fig. 1). Dogs 2, 3 and 4 lost respectively 4, 5·5 and 3·7 kg. Dog 1, the largest of the group, did not lose weight. It became clear after the initial few days that the severity of the systemic response to the drug was proportional to the dose/weight ratio. After 5 weeks, dog 2 began to lose its hair, and within a further 3 weeks, no hair remained on the face.

From the 6th to the 9th week the three animals which had lost weight began once more to gain. The animals least affected returned to a weight equal to or slightly above the pre-treatment level. The smaller, more severely affected dogs (2 and 3) failed to return to their normal weight while under treatment, but by the 14th week dog 2 had regained the lost hair.

Serum Proteins

The mean serum protein level (Fig. 1) remained significantly unchanged during the first 8 weeks, but then showed a sudden transient rise, falling again to the previous level within 5 weeks. Concurrently, changes in the albumen/globulin ratio occurred (Table); these were most severe in dogs 2 and 3, the animals most severely affected by the drug. In dog 2, between the 8th and 13th week there was a rapid change in the albumen/globulin ratio, which rose from 1·4 : 1 to 3·2 : 1, falling again to the earlier figure after 5 weeks. In dog 3, a similar type of change was delayed to the 15th week, reaching its maximum, 4·5 : 1, at the 23rd week, and falling again to normal at the close of the experiment. Dogs 1 and 4, which suffered less from the systemic actions of the drug, showed insignificant changes in the serum protein levels or ratios.

Electrophoretic pattern of serum proteins.—The patterns did not differ from normal.

Blood Changes

Haemoglobin.—Soon after the feeding of hydralazine began, falls in the haemoglobin values were observed (Fig. 1, Table). These closely paralleled the alterations in weight. Two animals showed an abrupt and steep fall in Hb. concentration during the early weeks of the experiment when both the changes in weight and the systemic disturbances were most severe. The lowest level was reached in dog 3, and subsequent increase was long delayed; in dog 2 recovery was more rapid, although there was a further fall between the 22nd and 26th week. The

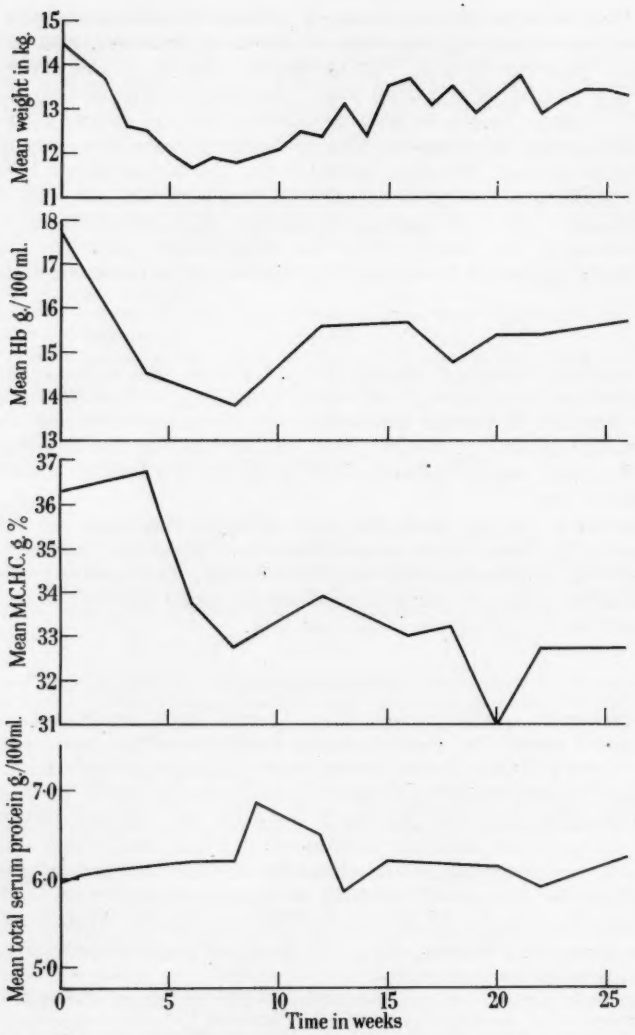
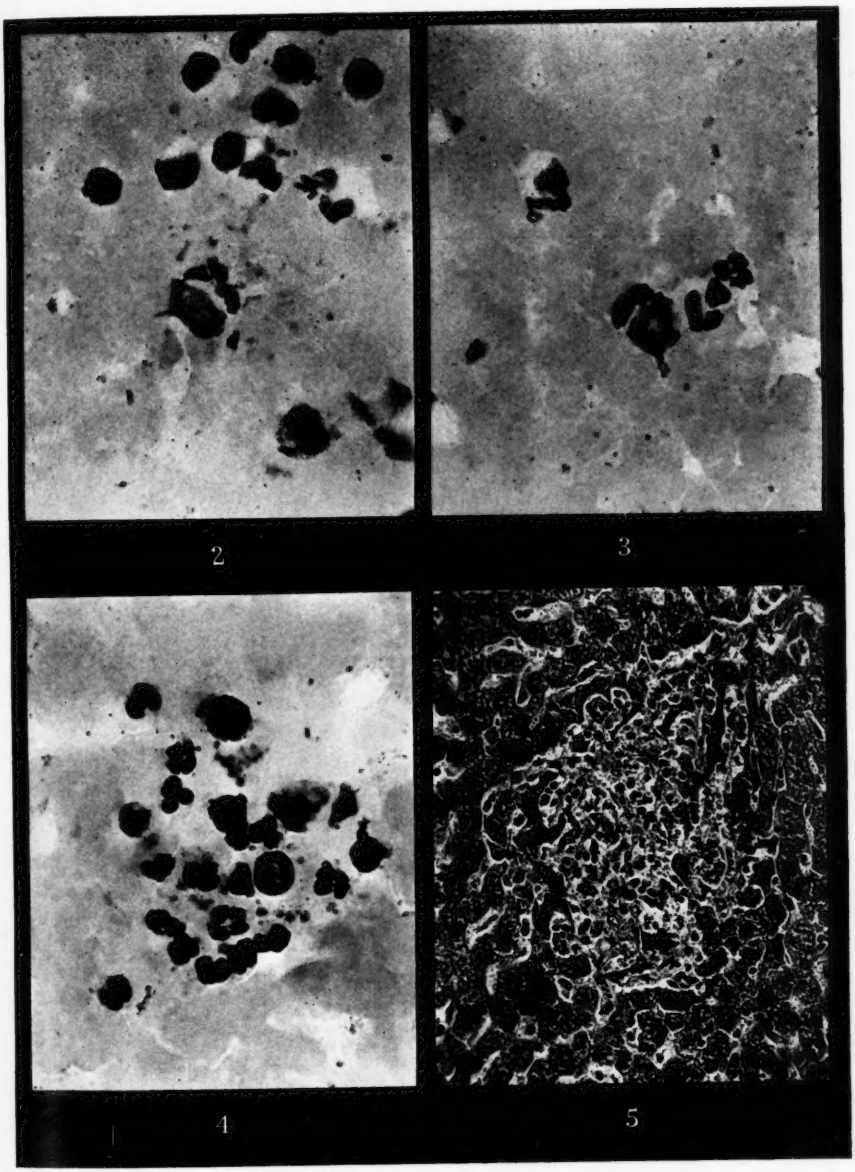


FIG. 1.—Effect of oral hydralazine on mean weight, haemoglobin, mean corpuscular haemoglobin concentration and serum protein of four dogs.

EXPLANATION OF PLATE.

FIG. 2 and 3.—Ingestion of basophilic nuclear material by polymorphonuclear leucocytes, after incubation of dog serum with normal human leucocytes for 2 hr. at 37°. (× 800.)
FIG. 4.—Cluster of polymorphonuclear leucocytes surrounding basophilic material, probably of nuclear origin. (× 800.)
FIG. 5.—Focal hepatic necrosis. (× 215.)



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TABLE—*Changes in Weight and Laboratory Findings during Hydralazine Treatment.*

	Time in weeks												
	0	3	6	9	12	15	18	21	24	27	30	33	36
Weight (kg)	70	70	70	70	70	70	70	70	70	70	70	70	70
Hemoglobin (g/100 ml)	14.5	14.5	14.5	14.5	14.5	14.5	14.5	14.5	14.5	14.5	14.5	14.5	14.5
Hematocrit (%)	43	43	43	43	43	43	43	43	43	43	43	43	43
Leukocytes (per mm³)	6,000	6,000	6,000	6,000	6,000	6,000	6,000	6,000	6,000	6,000	6,000	6,000	6,000
Differential count													
Eosinophils (%)	2	2	2	2	2	2	2	2	2	2	2	2	2
Stab neutrophils (%)	3	3	3	3	3	3	3	3	3	3	3	3	3
Segmented neutrophils (%)	60	60	60	60	60	60	60	60	60	60	60	60	60
Lymphocytes (%)	30	30	30	30	30	30	30	30	30	30	30	30	30
Monocytes (%)	5	5	5	5	5	5	5	5	5	5	5	5	5
Platelets (per mm³)	250,000	250,000	250,000	250,000	250,000	250,000	250,000	250,000	250,000	250,000	250,000	250,000	250,000
Urinalysis													
Protein	0	0	0	0	0	0	0	0	0	0	0	0	0
Glucose	0	0	0	0	0	0	0	0	0	0	0	0	0
Acetone	0	0	0	0	0	0	0	0	0	0	0	0	0
Sediment	0	0	0	0	0	0	0	0	0	0	0	0	0
Urea nitrogen (mg/100 ml)	12	12	12	12	12	12	12	12	12	12	12	12	12
Creatinine (mg/100 ml)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Uric acid (mg/100 ml)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Sodium (mEq/liter)	140	140	140	140	140	140	140	140	140	140	140	140	140
Potassium (mEq/liter)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Chloride (mEq/liter)	100	100	100	100	100	100	100	100	100	100	100	100	100
Bicarbonate (mEq/liter)	25	25	25	25	25	25	25	25	25	25	25	25	25
Glucose (mg/100 ml)	90	90	90	90	90	90	90	90	90	90	90	90	90
Total protein (g/100 ml)	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0	7.0
Albumin (g/100 ml)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Globulin (g/100 ml)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Bilirubin (mg/100 ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Alkaline phosphatase (units/100 ml)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Cephalin flocculation (units)	0	0	0	0	0	0	0	0	0	0	0	0	0
Thymol turbidity (units)	0	0	0	0	0	0	0	0	0	0	0	0	0
Prothrombin time (sec)	12	12	12	12	12	12	12	12	12	12	12	12	12
Serum transaminase (units/100 ml)	20	20	20	20	20	20	20	20	20	20	20	20	20
Lactic dehydrogenase (units/100 ml)	100	100	100	100	100	100	100	100	100	100	100	100	100
Electrocardiogram													
Rate	70	70	70	70	70	70	70	70	70	70	70	70	70
Rhythm	S	S	S	S	S	S	S	S	S	S	S	S	S
Axis	N	N	N	N	N	N	N	N	N	N	N	N	N
Intervals	N	N	N	N	N	N	N	N	N	N	N	N	N
Complexes	N	N	N	N	N	N	N	N	N	N	N	N	N
Chest roentgenogram	N	N	N	N	N	N	N	N	N	N	N	N	N

S = sinus; N = normal

TABLE—*Changes in Weight and Laboratory Findings during Hydralazine Treatment.*

	Time in weeks												
	0	2	4	6	8	9	12	13	15	18	20	22	26
Dog 1 (Male)													
Wt. in kg.	16·9	17·2	17·4	17·8	17·4	16·6	17·2	17·8	18·6	18·7	17·6	18·0	18·2
Hb. g./100 ml.	18·9	..	17·7	17·0	17·2	..	17·0	..	16·6	14·2	15·4	16·6	15·7
W.B.C./cu. mm.	10,400	..	9,500	14,600	17,200	..	18,600	..	12,000	11,200	..	12,800	..
P.C.V. per cent	55	..	51	49	51	..	48	..	52	42	52	50	47
E.S.R. mm./hr.	0	..	2	0	0	..	0	..	0	..	..	..	0
M.C.H.C. per cent	34·4	..	34·6	34·7	33·8	..	35·3	..	32·0	33·7	29·5	33·2	33·3
Plasma prot.	6·66	6·15	..	6·7	6·72	7·03	6·50	6·00	6·40	..	6·35	6·29	6·60
A/G ratio	1·29	1·1	..	1·2	1·34	1·13	1·89	2·17	1·5	..	1·64	1·53	1·12
L.E. cells	—	—	—	—	—	—	—	—	—	..	—	..	..
Dog 2 (Male)													
Wt. in kg.	11·0	8·6	7·4	7·0	7·0	7·8	8·5	9·2	9·8	9·2	9·4	9·0	9·2
Hb. g./100 ml.	19·2	..	12·4	13·9	14·9	..	16·6	..	16·0	15·4	15·7	16·1	14·1
W.B.C./cu. mm.	7,800	..	3,000	9,600	12,600	..	11,200	..	12,400	9,000	..	10,200	..
P.C.V. per cent	62	..	35	42	46	..	52	..	50	..	51	49	43
E.S.R. mm./hr.	0	..	1	1	0	..	0	..	0	..	..	..	0
M.C.H.C. per cent	31·0	..	35·5	33·0	32·5	..	31·9	..	32·0	..	30·7	32·9	32·7
Plasma prot.	5·80	6·14	..	5·61	6·14	7·10	5·75	5·53	5·67	..	5·61	5·80	5·81
A/G ratio	1·47	0·94	..	1·07	1·42	3·05	3·26	2·09	2·36	..	2·50	2·14	0·94
L.E. cells	—	—	—	—	—	+	+	+	..	..	—	..	..
Dog 3 (Female)													
Wt. in kg.	14·0	12·0	11·0	9·4	9·2	9·2	9·2	10·3	10·2	10·2	11·2	10·2	11·0
Hb. g./100 ml.	15·4	..	11·7	11·7	9·2	..	12·2	..	11·7	..	12·6	12·0	14·5
W.B.C./cu. mm.	11,400	..	2,750	8,200	13,400	..	8,200	..	4,600	8,800	..	..	..
P.C.V. per cent	39	..	32	34	27	..	34	..	33	35	40	37	46
E.S.R. mm./hr.	0	..	5	5	5	..	0	..	0	..	..	..	0
M.C.H.C. per cent	39·5	..	36·5	34·5	34·0	..	35·7	..	35·5	34·3	31·5	32·5	31·5
Plasma prot.	6·36	6·59	..	6·33	6·01	7·09	6·54	7·11	6·59	..	6·32	5·58	6·00
A/G ratio	1·39	1·49	..	1·20	1·10	1·00	1·58	1·45	1·86	..	3·73	4·50	1·22
L.E. cells	—	—	—	—	—	+	+	+	..	..	—	..	..
Dog 4 (Male)													
Wt. in kg.	16·2	16·0	14·2	12·6	13·6	14·0	14·8	15·0	15·6	16·0	15·3	14·6	15·0
Hb. g./100 ml.	17·2	..	16·2	14·2	13·8	..	17·7	..	16·6	17·7	18·0	16·9	18·6
W.B.C./cu. mm.	3,000	..	2,500	8,600	8,400	..	9,400	..	8,200	7,800	..	5,000	..
P.C.V. per cent	49	..	40	43	44	..	54	..	51	56	56	52	56
E.S.R. mm./hr.	0	..	1	0·5	0	..	0	..	0	..	..	..	0
M.C.H.C. per cent	35·0	..	40·5	33·0	31·0	..	33·0	..	32·6	31·5	32·1	32·4	33·3
Plasma prot.	5·33	5·38	..	6·11	6·04	6·35	7·21	4·89	6·15	..	6·27	5·93	6·54
A/G ratio	1·90	0·86	..	0·72	0·59	0·98	1·55	1·62	0·95	..	1·97	1·57	1·16
L.E. cells	—	—	—	—	—	—	—	—	..	..	—	..	..

Dog 5 (Control)

Wt. in kg. 17·5 W.B.C./cu. mm. 8300. E.S.R. 0 mm./hr. Plasma prot. 7·04 Albumin 4·43.
Hb. g./100 ml. 19·6 P.C.V. 58 per cent. M.C.H.C. 33·3 per cent. Globulin 2·61. A/G ratio 1·69/1·0.
L.E. cells *nil*.

changes in haemoglobin in dogs 1 and 4, the largest animals, were relatively slight.

Mean corpuscular haemoglobin concentration.—There was a progressive fall in the M.C.H.C. (Fig. 1). Unlike the other values the M.C.H.C. did not return to normal as the experiment progressed. In dogs 2 and 4, a transient rise preceded the fall.

Packed cell volume.—In each animal there was an early apparent fall in the P.C.V. Later these values returned to normal.

Erythrocyte sedimentation rate.—There was no significant change in the E.S.R. in any animal during the experiment.

Leucocytes.—The total leucocyte counts in three animals (2, 3 and 4) fell at the start of the experiment, but thereafter returned to normal and showed no further significant change. The differential leucocyte counts remained normal.

L.E. Preparations

Examination of the L.E. cell preparations revealed no cells of suggestive appearance until 9 weeks after the experiment began. Occasional cells were then found with an appearance resembling those illustrated by Comens (1956) (Fig. 2 and 3). Several were clearly polymorphonuclear leucocytes, with ingested basophilic material, probably of nuclear origin. In some the nucleus was displaced to one side by a relatively large inclusion. In most, possibly owing to the drastic method of preparation, the cytoplasm was indistinct. Few of the inclusions were completely homogeneous; some showed the appearances described as the late " Tart " cell (Weiss and Swift, 1955).

In addition to these cells, which morphologically seldom resembled the classical L.E. cell of Hargraves, Richmond and Morton (1948), there were moderate numbers of basophilic bodies lying either discretely in the film, or surrounded by varying numbers of polymorphs, apparently engaged in phagocytosis (Fig. 4). The classical rosettes of D.L.E. were not recognized. As the condition of the animals improved, it became less easy to identify the abnormal cells. Incubation of the serum of dogs 1 and 4 with normal human leucocytes did not at any time result in the production of the abnormal cells, or of cells of the " Tart " cell type.

Histology

Examination of the kidneys from each dog confirmed the presence of glomerular capillary loops thicker and more dilated than those seen in normal human glomeruli. The appearances were similar to those described by Comens (1956). The amount of P.A.S.-positive material did not differ from that in the glomeruli of the control dogs, but glomerular capillary walls were thicker in older than in young animals.

Scattered throughout the liver of dogs 1, 2 and 3, and less prominent in the liver of dog 4, were numerous small focal necroses (Fig. 5).

The spleens from the treated animals were large, but there was no sign of " onion-skin " fibrous perivascular cuffing.

The lungs from dogs 1, 2 and 4 were entirely normal. Scattered over the pleural surfaces of the lungs of dog 3 were numerous small cholesterol granulomata.

Examination of sections of the lymph nodes, skin, heart, intestine, ovary or testis, suprarenal, thyroid, muscle, pancreas, synovia and bone marrow

revealed no changes which could be regarded as significant, and none which in any way resembled those found in D.L.E.

DISCUSSION

The continued administration to healthy dogs of large doses of hydralazine resulted in acute, severe and prolonged illness. The early claims of Comens (1956) have thus been confirmed, and it would appear that a condition has been produced in dogs closely resembling that which may occur in humans after treatment with hydralazine.

Nevertheless, it remains uncertain whether the experimental hydralazine syndrome bears any relationship to D.L.E. The diagnosis of D.L.E. depends on the demonstration of a characteristic clinical picture, of a positive L.E. cell test, or of typical histological changes in the organs at biopsy or necropsy (Harvey, Shulman, Tumulty, Conley and Schoenrich, 1954). The question arises : to what extent do the changes found in the experimental hydralazine syndrome conform with these criteria?

The principal reason for suggesting a link between D.L.E. and the experimental hydralazine syndrome (Comens, 1956) was the ability to demonstrate with sera from treated animals the appearance of cells morphologically resembling those which can be produced with sera from cases of D.L.E. (Hargraves *et al.*, 1948). In the experiments of Comens, and in those reported here, cells have been found which, indeed, superficially resemble those of D.L.E. The cells cannot be produced with sera from normal dogs, and are therefore a manifestation of the hydralazine syndrome. The morphology of the cells found in the present experiment, however, is not regarded as sufficiently characteristic to warrant the diagnosis of D.L.E.: the inclusion bodies are neither homogeneous, nor free from recognizable nuclear structure. They appear to resemble those shown in the paper of Comens (1956). It is therefore doubtful to what extent the experimental demonstration of these cells can be held to substantiate his claim to have reproduced D.L.E.

Those histological changes claimed by Comens (1956) to have resulted from the prolonged administration of hydralazine and to have confirmed the simulation of D.L.E. were confined to the kidneys. They included fibrinoid degeneration and prominence of the juxtaglomerular apparatus. Acidophilic hyaline thickening of the basement membrane of the glomerular capillaries, with the production of the " wire-loop " appearance, was also described. The present experiment has confirmed the presence, in freshly-fixed post mortem tissue collected immediately after death, of appearances similar to those found by Comens in the glomerular capillaries of less fresh material; these structures however, have been indistinguishable from those of untreated control animals. Fibrinoid change has not been observed and the juxtaglomerular apparatus has been no more prominent than in the normal dog.

Certain lesions in our animals have not previously been described as a result of the feeding of hydralazine to dogs. The most conspicuous of these has been the presence of many small multifocal necroses in the liver. These necrotic foci appeared to be only a few days old. It seems possible that repeated episodes of this type of necrosis may have occurred throughout the period of treatment, and that they may have been associated with metabolic disturbances, such as those occurring in the serum proteins, during the course of the illness. The appearance of these

focal necroses is of some interest in view of the suggestion by Mackay, Taft and Cowling (1956) that liver damage may act as a stimulus to the formation of LE cells in the human.

A rise in the globulin fraction of the serum is a characteristic finding in D.L.E. In Comen's animals, an increase in the α_2, β, and γ-fractions of the serum globulin was observed. In the present experiment, the electrophoretic pattern did not change significantly. The predominant trend during the development of the syndrome was for albumen to increase, and for globulin to fall, with little change in the total serum protein.

The development of iron deficiency anaemia has characterized the use of hydralazine in human disease. This has been confirmed experimentally. Although the haemoglobin level tended to return towards normal as treatment progressed, iron deficiency of the red cells persisted.

The mechanism by which weight loss occurs remains uncertain. The administration of the drug in capsules largely excludes taste as a factor in causing malnutrition. During much of the experiment the animals ate voraciously. The iron deficiency anaemia which is a prominent sign of the disease is probably caused by the combination of hydralazine as a chelating agent with iron in the gut, and it is possible that the combination of the drug with some other essential metabolite, in a manner analogous to its union with iron, could disturb protein metabolism, and thus cause loss of weight.

The significance of the changes interpreted by Comens as confirming the reproduction in dogs of D.L.E. remains uncertain. Although the experimental hydralazine syndrome is an entity of much interest, its relationship to D.L.E. cannot be accepted as proven.

SUMMARY

The feeding to healthy dogs of large amounts of L-hydrazinophthalazine caused acute illness characterized by loss of appetite, loss of weight, anaemia and sometimes convulsions; its course was unrelated to the taste of the drug.

There were changes in the plasma proteins, and in suitable preparations cells were found which resembled those of D.L.E.; they were not sufficiently characteristic to be diagnostic of that disease.

The illness did not progress to death, but appeared to become static. The red cells remained iron-deficient, but partial recovery of lost weight and re-growth of lost hair suggested that spontaneous recovery had begun.

The animals were killed after 26 weeks and the tissues examined in detail. The changes found included multifocal hepatic necrosis and certain incidental abnormalities. No lesions resembling those of D.L.E. were discovered which were not also found in control animals.

It is concluded that the prolonged administration of L-hydrazinophthalazine to dogs causes a syndrome which resembles that found in humans suffering from overdosage with this drug, but which bears no clear relationship to naturally occurring D.L.E.

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